

Topoisomerase II of the Malarial parasite.

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1998

(i)



Abstract of Thesis.

The gene encoding topoisomerase II of *P. falciparum* (PfTopoII) was isolated and characterised in 1994 (Cheesman *et al.*) as part of an overall strategy to isolate parasite genes involved in DNA replication and evaluate their potential as anti-malarial drug targets. As a natural progression, this study has focussed on heterologous expression of the gene to enable sufficient recombinant protein to be purified for biochemical analysis and drug screening. The species divergent C-terminal region of the protein was expressed in *E. coli*, and used to raise polyclonal antiserum in rabbits, which detects a protein of 160/175 kDa in extracts from *P. falciparum*.

The same antiserum was used to monitor expression of recombinant PfTopoII in baculovirus. A triplet of proteins of 160/175 kDa were detected by the antiserum in cell extracts derived from baculovirus-infected insect cells, but in very small quantities. This is close to the predicted size of PfTopoII. Incorporation of a C-terminal hexahistidine affinity tag into the protein was used as a means to purify the small amount of recombinant protein that was made, using nickel affinity chromatography. Decatenation assays, which are a specific test for type II topoisomerase activity, detected activity in some fractions obtained from the affinity purification. However, a mock purification, using an unrelated recombinant baculovirus gave similar results, indicating that a contaminating co-purifying topoisomerase II activity was present.

Full length expression of recombinant PfTopoII was not detected in cell extracts prepared from *E. coli*. However, there is evidence that the recombinant protein was expressed, but may have undergone rapid proteolytic degradation *in vivo*. Expression of a functionally inactive version of the protein, in which the active site, tyrosine, was changed to phenylalanine, had no noticeable affect on protein stability.

A modified version of the PfTopoII gene, in which the first four codons were replaced with the first five codons of the yeast topoisomerase II gene was expressed in *S. cerevisiae*. A control plasmid expressed the human topoisomerase II α homologue to high levels, but no evidence of

expression of recombinant PfTopoII was detected. Northern analysis of the mRNA revealed the presence of truncated transcripts, and RT-PCR mapped the sites of polyadenylation to 370 and 376 nucleotides from the AUG. An analysis of the region upstream of these sites revealed the presence of specific AT-rich sequence elements which are known to cause transcription termination of yeast mRNAs. Site directed mutagenesis was used to change these sequences, and analysis of the mRNA from the modified construct showed that the original transcriptional block had been abolished, but was replaced by a new transcriptional block, which gave rise to a truncated transcript some 617 nt in length.

An investigation into the developmental regulation of PfTopoII throughout the blood stages of parasite growth was also undertaken. Northern analysis indicates that three transcripts accumulate principally in the trophozoite stage, although interestingly, work conducted by Paul Horrocks indicates that the promoter is active in both ring and trophozoite/schizont stage parasites. Western analysis of protein extracts derived from synchronised populations of parasites shows that trophozoite and schizont stages express a triplet of proteins of 160/175 kDa in size, and at similar levels. Although protein extracts from trophozoite and schizont stage parasites are proficient at decatenation, activity was highest in schizonts, and could be inhibited completely after immunodepleting the parasite extracts.

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Acknowledgements

I am indebted to *Robert Ridley* who introduced me to the fascinating but frustrating Malaria parasite. I am also extremely grateful to *Brian Kilbey* without whose help, encouragement, advice and support, the work contained within this thesis would not have been possible.

I wish to thank the members of the *Kilbey Group*, past and present for their support. In particular, *John White, Jill Douglas, Kerrie Tosh, Paul Horrocks and Ali Allouèche*. I would like to express my thanks also to *Ann-Marie Ketchen* for technical assistance with parasite culture, and to *Jean Ramsey* and *Joan Smail* for their help. Thanks also go to the Photography Department.

Some of this work would not have been possible without the generosity of *Caroline Austin* who supplied some of the plasmids and strains used for the yeast expression work, and whose advice was highly valued. The work presented here was supported by the Medical Research Council.

I would like to dedicate this thesis to *Walter*, my husband, to thank him for his support, patience and understanding. Thanks also go to very special members of my family, *Mum and Derek, Emma and Leanne*.

Abbreviations.

A	Adenosine
Amp	Ampicillin
ATG	Initiating methionine
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
CCC	Covalently closed circular
cDNA	Complementary DNA
CIP	Calf intestinal alkaline phosphatase
cm	Centimetre
DAPI	4'-6'-diamino-2-phenylindole
dATP,	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddATP	Dideoxyadenosine triphosphate
ddCTP	Dideoxycytidine triphosphate
ddGTP	Dideoxyguanosine triphosphate
ddTTP	Dideoxythymidine triphosphate
dGTP	Deoxyguanosine triphosphate
DMF	Dimethylformamide
DNaseI	Deoxyribonuclease I
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
EDTA	Diaminoethanetetra N, N, N', N'-tetra-acetic acid
EGTA	Ethylene glycol-bis (β -aminoethyl ether) N, N, N', N'-tetra-acetic acid
DHFR	Dihydrofolate reductase
DHFR-TS	Dihydrofolate reductase-thymidylate synthetase
FBS	Fetal bovine serum
g	Gram
GST	Glutathione S-transferase
GyrA	Gyrase A

GyrB	Gyrase B
ICMB	Institute of Cell and Molecular Biology
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IPTG	Isopropyl-1-thio- β -D-galactoside
kb	Kilobase
KCl	Potassium chloride
kDa	Kilodalton
KDNA	Kinetoplast DNA
l	Litre
LB	Luria Bertani medium
mCi	Microcurie
MCS	Multiple cloning site
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
MOPS	Morpholinepropanesulphonic acid
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
OC	Open circular
occ ⁻	Occlusion body negative phenotype
occ ⁺	Occlusion body positive phenotype
OD	Optical density
ORF	Open reading frame
OV	Occluded virus
p	Prefix for plasmid DNA
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pf	<i>Plasmodium falciparum</i>
<i>pfmdr 1</i>	<i>Plasmodium falciparum</i> multi drug resistance 1 gene
pfu	Plaque forming unit

p.i	Post infection
PMSF	Phenylmethanesulphonyl fluoride
Pol α	Polymerase alpha
Pol δ	Polymerase delta
RNA	Ribonucleic acid
RPMI	Rosewell Memorial Park Institute
sdH ₂ O	Sterile distilled water
SDS	Sodium dodecyl sulphate
Sf9 cells	<i>Spodoptera frugiperda</i> 9 cells
SSC	Standard saline citrate
SV40	Simian Virus 40
T	Thymine
TAA	Translational stop codon
TBE	Tris borate EDTA
Temed	N,N,N',N'- tetraethylmethylenediamine
TOP2	Topoisomerase II gene
TopoI	Topoisomerase I
TopoII	Topoisomerase II
TopoIII	Topoisomerase III
TopoIV	Topoisomerase IV
Tris-HCl	Tris hydrochloride
UV	Ultraviolet (light)
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

λ	Lambda phage
μ g	Microgram
μ l	Microlitre
μ M	Micromolar
$^{\circ}$ C	Degrees Celsius
%	Percent

Chapter 1

1.1 Introduction.

Estimates suggest that between 1.5 and 2.7 million people die annually from the disease Malaria, and the number of infected individuals is currently 300 to 500 million (World Health Organisation, 1994).

In humans, the disease is caused by a group of protozoan parasites of the genus *Plasmodium*, which fall within the phylum Apicomplexa, grouped together on the basis of the presence of an apical complex at some stage of the life cycle. Malaria is an insect-borne infection caused by the bite of an infected *Anopheles* mosquito. Only female *Anopheles* are able to transmit the malaria parasite and transmission occurs upon ingestion of a blood meal which is necessary for successful egg development in the female.

There are four species of *Plasmodium* known to infect man, *Plasmodium vivax*, *P. ovale*, *P. malariae*, and *P. falciparum*. Of these species, *P. falciparum* is the most dangerous, because it can lead to the potentially lethal condition, cerebral Malaria. Young children, pregnant women, foreign travellers and military troops on active service in endemic areas are most at risk from the disease.

40% of the world's population now live with endemic Malaria, but twenty years ago this percentage was significantly lower, perhaps closer to 32%. Several decades ago it was believed that Malaria would eventually share the same fate as Smallpox and be effectively controlled, if not completely eradicated. However, Malaria eradication has only been successful in the more temperate regions of the globe.

Despite extensive mosquito eradication programmes by the WHO as far back as 1955 using insecticides such as DDT, malaria is present today in 102 countries. The disease has profound economic consequences for developing third world countries, where the cost of disease is both financial and humanitarian.

The disease is confined to tropical and sub-tropical zones including Central Africa, Thailand, India and South America. The native people of malarious regions that survive infection develop premunition which is a state of partial immunity where parasite multiplication is decreased but blood parasitaemia still exists. Repeated infection maintains an

equilibrium of asymptomatic infection. Thus malaria can be seen as an insidious problem where symptomless infected individuals contribute to a reservoir of infection between the mosquito vector and its host.

The primary control strategy for Malaria is geared towards chemotherapy, however, many of the drugs which are commonly used for treatment, such as chloroquine, are now virtually obsolete due to the emergence of drug resistant strains. So serious is the problem in many areas of the world that *falciparum* malaria is now almost untreatable. Not only is it imperative that new drug treatments are developed, but if we are to gain effective long term control of the disease we must also strive to achieve a better understanding of how novel drugs operate.

In this introduction, traditional and modern antimalarial drug treatments will be considered, along with novel antimalarial drugs and vaccines which are still under development.

1.2 Lifecycle of the Malarial parasite in man.

The lifecycle is shown in full in Figure 1 (Philips, 1983). It consists of two distinct phases. The exogenous sexual phase (sporogony) which takes place in certain *Anopheles* mosquitoes, and the endogenous asexual phase (schizogony) that occurs in the vertebrate host.

1.2.1 The mosquito host.

The cycle commences upon ingestion of a blood meal containing infected red blood cells harbouring male and female gametocytes from the human host. Male gametocytes undergo three successive rounds of DNA replication resulting in octoploidy prior to maturation. Eight motile male microgametes are produced by exflagellation and this restores the haploid state.

Female gametocytes ingested in the blood meal shed the red blood cell and differentiate into mature macrogametes. Meiosis occurs soon after fertilisation by the microgametes. Approximately 18-24 hours later the zygote elongates, becomes motile and is known as an ookinete form which is able to penetrate the mid gut epithelium to the outer surface where it undergoes redifferentiation to produce the oocyst.

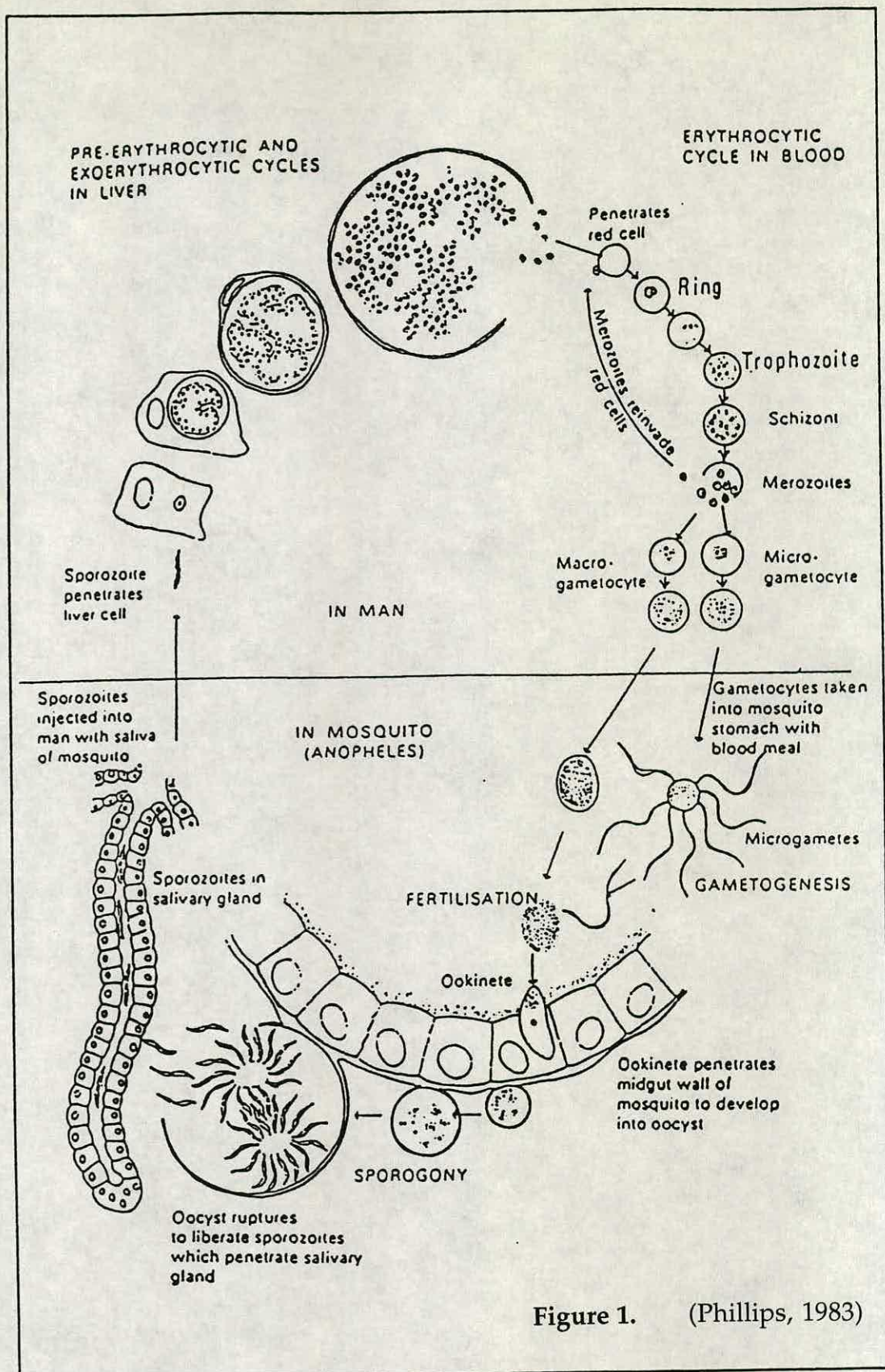


Figure 1. (Phillips, 1983)

Growth and repeated cell division eventually result in thousands of mature nucleated sporozoites which rupture the oocyst and migrate to the salivary glands via the body cavity, thus completing the infection process in the mosquito.

1.2.2 The human host.

Infective sporozoites from the anopheline salivary glands are released into the human circulation upon ingestion of a blood meal. Sporozoites that are not destroyed by phagocytosis enter the parachymal cells of the liver where they mature and undergo multiplication. This is known as exo-erythrocytic schizogony and it lasts for around 6-16 days. The end of this period is characterised by rupture of the mature schizont, releasing infective merozoites that invade the red blood cells. This is the start of the erythrocytic cycle, the first developmental stage of which is the ring form. Continued parasite growth and development give rise to the trophozoite form that consumes host cell haemoglobin for nutrition. Mature trophozoites then undergo asexual cell division to produce schizonts which rupture the red cell blood cell membrane releasing invasive merozoites which can reinvade uninfected erythrocytes in the circulation. This repetitive cycle is highly characteristic of a Malaria infection and is responsible for the major clinical symptoms of the disease.

The intraerythrocytic cycle varies in length according to the species of Plasmodium responsible for the infection. It is, for example, completed in around 48 hours in *P. falciparum*. Because the cycle is repeated over and over again, a very high blood parasitaemia eventually develops. After many generations, sexually differentiated forms (gametocytes) are produced facilitating completion of the sexual cycle in the anopheline vector following transmission (Bruce-Chwatt, 1985)

1.3 Drugs used in the treatment of Malaria.

A number of clinically important drugs are available that target the parasite. A description of some of the most well known and widely used is given below.

1.3.1 The chinchona alkaloids.

Quinine and related antimalarials.

Long before the causative agent of Malaria was positively identified, South American Peruvian Indians are reported to have chewed the bark of the Chinchona tree to relieve 'bad fevers' which were probably caused by Malaria. Some time later, 16th century Jesuit priests, recognising the medicinal properties of the bark, transported it back to Spain where it was used to treat Malaria that was prevalent in Europe at that time.

Today, quinine is still used to treat cerebral Malaria, but although cheap to administer, it is only used as an emergency treatment due to its high toxicity in man. One of the most common side effects is deafness and tinitis, symptoms that were frequently experienced by British military troops engaged in active service in malarious regions during the First and Second World Wars.

There are two principal pharmacological activities present in Chinchona bark, quinine and quinidine, both of which contain a quinoline ring structure that was used as the basis of the synthetic derivatives, chloroquine, amodiaquine, mepacrine and sonetaquine manufactured during the 1930's and 40's. After the Second World War chloroquine came into widespread use and, motivated by the possibility of complete eradication of the disease, was used as a population prophylactic in Thailand and South America. It was in these countries where drug resistance was first reported (Foote and Cowman, 1994).

Quinoline-containing antimalarials appear to target the asexual blood stages of the parasite that degrade haemoglobin as a source of nutrition. The parasite food vacuole is the site of degradation. Here, haemoglobin is broken down to its constituent amino acids and heme, in the form of ferriprotoporphyrin IX (FP), which is toxic to the parasite. As a result the parasite sequesters it as hemozoin, a non-toxic crystalline structure composed of insoluble haem polymers (Slater *et al.*, 1991). Chloroquine becomes concentrated in the food vacuole of the parasite and microscopic studies have shown that it induces gross morphological changes in the organelle; similar results have been obtained for another quinoline compound, mefloquine, which was developed by the Walter Reed Army

Research Institute in response to the large number of cases of malaria amongst American troops during the Vietnam conflict.

The fact that chloroquine accumulates 100-fold more in parasitised erythrocytes is believed to be due to its weak basicity, which allows it to diffuse through membranes. Once chloroquine passes into the acidic food vacuole of the parasite, it becomes deprotonated and is unable to pass out again (De Duve *et al.*, 1974). The precise mechanism of its action remains uncertain, however, and several possibilities exist, including possible inhibition of enzyme activity within the food vacuole (Vander, 1986). The most popular explanation at present is that chloroquine renders the parasite incapable of detoxifying haem causing the build up of FP which is toxic. There has been much debate about the possibility that a haem polymerase activity exists in the food vacuole. Evidence in favour of this comes from Slater and Cerami, (1992), and evidence against from Egan *et al.*, (1994), who found evidence of spontaneous polymerisation.

Chloroquine is a safe, effective antimalarial that is also extremely cost effective to administer (50p per person/week). However, *P. falciparum* parasites in particular have developed mechanisms to counteract its effects. One of these, has been shown to be an energy-dependent process in which chloroquine is expelled 40-50 times faster in chloroquine resistant parasites. Some drugs, such as verapamil, a calcium channel blocker, can reverse this process (Martin *et al.*, 1987). This efflux phenomenon is similar to the multi-drug resistant (MDR) phenotype found in mammalian tumor cells which also involves the rapid efflux of an array of chemically distinct anti-cancer drugs mediated by an ATP-dependent P-glycoprotein (Endicott *et al.*, 1989). In mammalian cells these proteins are encoded by *mdr* genes which are often amplified in MDR tumor lines resulting in large increases in P-glycoprotein levels. *P. falciparum* has two P-glycoprotein homologues, Pgh-1 and Pgh-2 which are encoded by two genes *pfmdr1* and *pfmdr2* (Foote *et al.*, 1989 and Wilson *et al.*, 1989). The Pgh-1 molecule is situated on the surface of the acid vesicle where its function appears to be related to regulation of the pH inside the food vacuole. Some chloroquine-resistant parasite lines are

found to have amplification of the *pfmdr 1* gene copy number (Foote *et al.*, 1990a), and there is strong correlation between amplification and overexpression of the Pgh-1 protein.

1.3.2 The antifolates.

Pyrimethamine, proguanil and sulphadoxine.

DNA synthesis is an essential requirement of all living creatures at some point in their life cycle, and the malaria parasite which reproduces asexually in its human host is no exception. Malarial parasites scavenge purine bases from the host red blood cell but synthesise pyrimidine bases *de novo*. The synthesis of the amino acids serine and methionine, in common with pyrimidines, require folate as a co-factor. The parasite pathway for folate production is targeted by four groups of drugs. Sulphadoxine and dapsone inhibit dihydropteroate synthetase (DHPS) which catalyses the conversion of p-aminobenzoic acid (PABA) to 7,8, dihydropteroate. Pyrimethamine and proguanil both act by binding to and inhibiting the activity of dihydrofolate reductase (DHFR) which catalyses the reduction of dihydrofolate to tetrahydrofolate.

As with chloroquine, resistance to pyrimethamine and sulphadoxine has developed and there is evidence that suggests that it is mediated by changes in the enzyme which reduce its affinity for the drug. Several changes have been mapped, for example, at position 108 serine is replaced by asparagine, giving a ten-fold increase in resistance (Cowman *et al.*, 1988).

Amplification of the DHFR gene has also been shown to increase levels of the enzyme (Inselberg and Zhang, 1988), however, this type of change has never been connected with pyrimethamine resistance in the field.

1.3.3 The sesquiterpenes.

Quinghaosue and its derivatives.

In Thailand, Cambodia, Vietnam and Burma the Malaria parasite is now resistant to chloroquine and virtually all other antimalarials including mefloquine (Larium) and halofantrine. Although health care is sufficiently well developed in Southeast Asia to keep the death rate

relatively low, there is now considerable concern that effective antimalarials are so limited.

One promising candidate, however, is the endoperoxide Quinghausue, (also known as artemisinin) which is extracted from the shrub *Artemisia annua* (wormwood). It has been used in traditional Chinese medicine as a cure for fever, Malaria and haemorrhoids for many centuries. Isolation of the active compound Quinghausue, was achieved in 1971 by the Chinese. Since then various semi-synthetic derivatives have been developed including artemether, arteether, artesunate and artelinate. These second generation drugs are now widely used in Thailand, Vietnam and China. Literally hundreds of synthetic endoperoxides have been developed in an attempt to improve the inherent short half-lives and poor solubility of these drugs, and also to avoid the high levels of recrudescence that occur following treatment.

Artemisinin and its derivatives are stage specific, targeting late ring stage and trophozoite stage parasites. They are also gametocidal, but do not affect the liver stages of the parasite and therefore cannot be used prophylactically. The cytotoxicity of artemisinin is mediated by a two step mechanism involving an endoperoxide bridge. In the first step, artemisinin is activated by heme or molecular iron giving rise to electrophilic alkylating intermediates and free radicals. In the step that follows, these highly reactive species react with and damage specific membrane associated proteins in the parasite (Meshnick, 1994). These drugs have been successfully used against chloroquine-resistant falciparum Malaria and are an effective way of treating the often fatal condition, cerebral malaria.

1.3.4 9-anilinoacridines.

Pyronaridine.

Pyronaridine, a 9-anilinoacridine derivative based on m-AMSA (figure 2) is another drug developed in the People's Republic of China during the 1970's, and is the product of 30 years chemical research which began with the mepacrine nucleus, selected on the basis of the search for a chloroquine alternative. Synthesis of a large number of derivatives

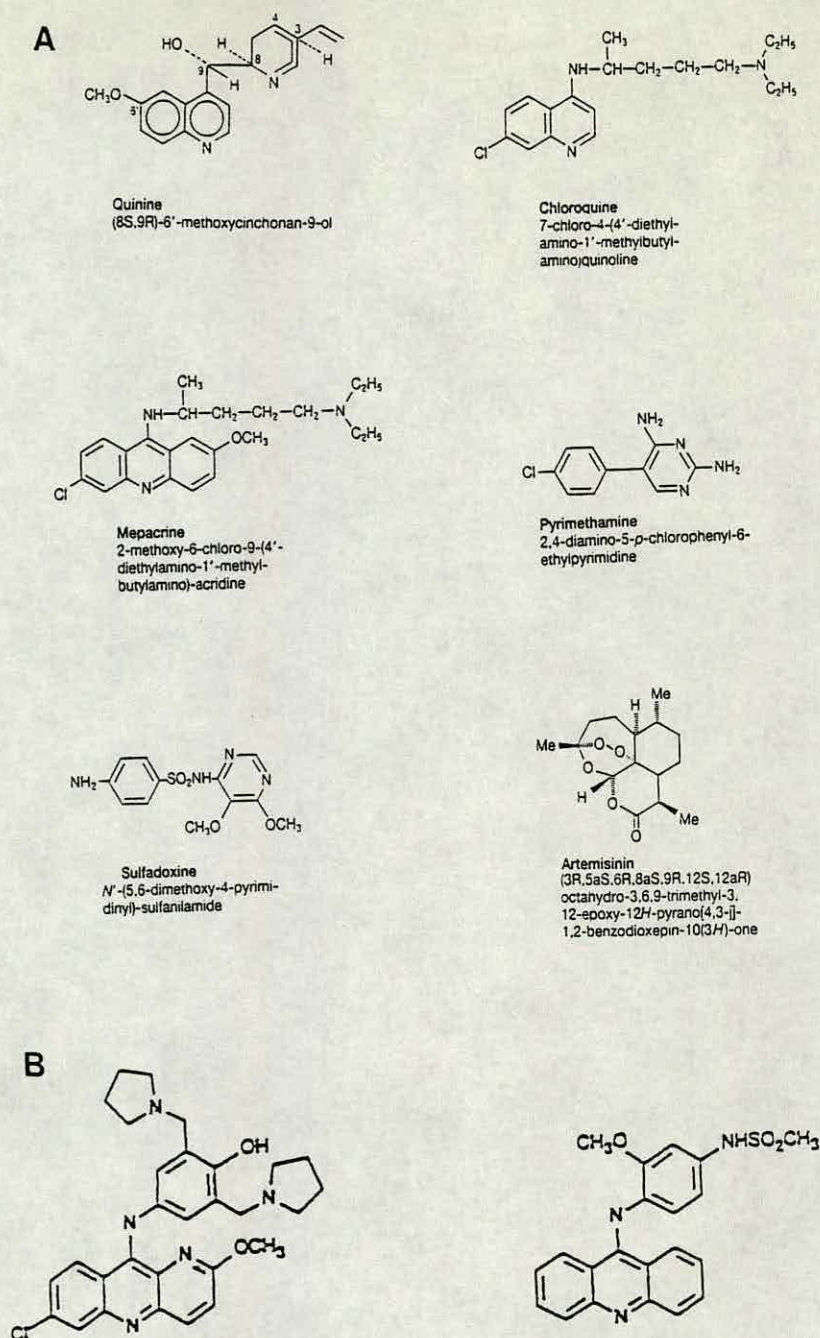


Figure 2. (a) Chemical structures of representative compounds from the major classes of antimalarial drugs discussed in the main text. (b) Structure of 9-anilinoacridine compounds pyronaridine, an antimalarial and m-AMSA (amsacrine) an anticancer agent.

produced a compound containing an amodiaquine side chain to the mepacrine nucleus. This derivative is a highly active schizonticide with few reported adverse effects (Fu and Xiao, 1991). The World Health Organisation have shortlisted pyronaridine as a potential candidate antimalarial whose use should be thoroughly evaluated outside of China (Olliaro and Trigg, 1995). To date, pyronaridine has undergone two successful clinical trials in Cameroon (Ringwald *et al.*, 1996) and Thailand (Looareesuwan *et al.*, 1996) with patients afflicted by acute uncomplicated Malaria.

Although clinical trials are underway, the target of this drug remains uncertain. Kawai *et al.*, (1996) have monitored the effects of pyronaridine on the *P. falciparum* parasitised red blood cells of a severely ill owl monkey, and found that it induced distinct morphological changes in the food vacuole of late trophozoite and schizont stage parasites, thirty minutes after administration. They concluded that pyronaridine must therefore interfere with the parasite digestive system.

Chavalitshewinkoon *et al.*, 1993, and Gamage *et al.*, 1994 have tested a range of 9-anilinoacridine derivatives against the pyrimethamine-chloroquine resistant KI isolate of *P. falciparum*.

The ability of these drugs to inhibit parasite growth in culture and topoisomerase II activity were tested. In the former study, blood stage parasites were incubated with drugs for 24 hours, followed by the addition of ^3H hypoxanthine for a further 24 hours. Drug activity was then recorded as the concentration of drug required to inhibit the incorporation of ^3H hypoxanthine into parasites by 50% (IC_{50}) compared to untreated controls. Human Jurkat leukemia cells were used for comparison, and cytotoxicity was measured in this case by assessment of cell growth (IC_{50}) and viability. The outcome of these experiments showed that some 9-anilinoacridine derivatives, including pyronaridine, appeared to exhibit selective antimalarial activity. There also appeared to be a relationship between antimalarial activity and enzyme inhibition. For example, one derivative called 7n, exhibited a potent antimalarial activity (in the tritiated hypoxanthine assay), and inhibited partially purified TopoII from the parasite and human Jurkat cells at

concentrations of 6 μ M and 20 μ M, respectively. However, it was recognised that inhibition of the parasite topoisomerase II enzyme may not be the sole mechanism of action (Gamage *et al.*, 1994). Clearly, further investigation is required to corroborate the promising preliminary studies that have been done with pyronaridine, if it is to be used effectively in the field as an antimalarial.

1.4 Vaccine development.

1.4.1 Conventional vaccines.

Considerable time, money and effort have been invested in attempting to obtain a safe and effective anti-malarial vaccine. Advances in this area have been discouragingly slow, however.

Plasmodium surface proteins should provide suitable targets for vaccine development, but a major problem relates to the fact that the parasite spends much of its life cycle within the cells of its host (eg. liver stages, ring, trophozoite, schizont and gametocyte blood stages) and thus "shielded" from the immune system. This is exacerbated when red blood cells infected with *P. falciparum* parasites become sequestered in the capillary systems of organs (Hyde, 1990).

The search for protective antigens has also been complicated by the discovery of numerous cross-reactive parasite proteins, which, it has been proposed, will impair the development of protective immune responses in the host. This may occur by absorption of antibodies to antigens that are not essential to parasite survival, or by induction of low affinity antibodies that are ineffective at directing a strong immune response. The latter mechanism has been termed the "smokescreen effect", and there is good evidence to support it. The antibody response to sporozoites, for example, is almost entirely directed towards the repetitive domain of the circumsporozoite protein, indicating that this appears to be a single immunodominant epitope.

Despite these problems, recombinant circumsporozoite surface protein/hepatitis B surface antigen fusions were recently found to protect six out of seven vaccinees in a preliminary trial (Stoute, 1997).

Another potential vaccine candidate developed by Columbian scientist

Manual Patarroyo, is currently undergoing clinical trials. It is a synthetic peptide vaccine based on the pre-erythrocytic and asexual blood stage antigens of *P. falciparum*. (SPf-66 vaccine).

Clinical trials of the vaccine began in 1985/1986 with monkeys and showed that the vaccine gave partial protection and was safe (Maurice, 1995). Since that time a number of trials have been conducted in humans with varying success. The best results came from a trial conducted in Tanzania, an area of intense perennial transmission of *P. falciparum*; children in the age group 1-5 were the subjects of the trial. The vaccine was shown to be highly immunogenic with all recipients positive after 3 doses. The efficacy was reported to be 31% (Alonso *et al.*, 1994).

Much criticism has been directed against the design of the early clinical trials for this vaccine and the most recent trial showed a disappointingly low level of protection (8%). Patarroyo now proposes to conduct a new trial in the summer of 1997 using fifty Columbian volunteers and a new adjuvant code named QS21 which has been shown to increase protection.

1.4.2 Transmission blocking vaccines.

Transmission blocking vaccines work by inhibiting development of the sexual stages of the parasite in the mosquito, so that transmission back to the human host cannot occur. The process is mediated by vaccinating the host with antibodies directed against specific parasite sexual stage antigens. In this sense, the vaccine is said to be altruistic because the human host gains no direct protection or immediate benefit from the vaccine. Various sexual stage antigens targets have come under scrutiny, most of which are present on the surface of the parasite, for example Pfs230, a gametocyte-gamete surface antigen and Pfs25, a ookinete surface antigen.

The concept of transmission blocking immunity is not a new idea and early work using whole gametes (Carter and Chen, 1976) and a mixture of macrogametes and gametes (Carter *et al.*, 1979) has shown that the transmission route between host to vector can be interrupted. This has also been tested with monoclonal antibodies using a membrane feeding apparatus, which mimics what might occur when mosquitoes feed on an

Malaria infected individual.

No hard data is available at present on how well these vaccines might perform in the field, however.

1.5 Topoisomerases.

The search for novel antimalarial drug targets has recently focussed on a group of enzymes which are better known as the cellular targets of many widely prescribed anticancer and antimicrobial drugs. These enzymes, the topoisomerases, perform many roles relating to DNA topology within the cell, for example, DNA replication, transcription and recombination, as well as chromosome structure and segregation.

Before the biological roles that the topoisomerases play is discussed, however, certain features relating to the topological organisation and characteristics of a DNA molecule require definition. The linking number of a DNA molecule is defined as the number of times that the two strands of a DNA helix are interwound in a covalently closed DNA molecule. The mean helical pitch of unconstrained DNA is 10.6 base pairs per turn and a relaxed covalently closed circular DNA will have a linking number equal to an integral value close to $N/10.6$, where N is the number of DNA in base pairs (Wang 1979). It follows that underwound DNA circles which are negatively supercoiled will have linking numbers less than this value, whilst overwound DNA circles which are positively supercoiled will have linking numbers greater than this value. The only way to change the linking number of a DNA circle is by breaking one or both of the DNA strands so that the interwinding of the two strands is altered. Identical DNA molecules which differ only in their respective linking numbers are referred to as topological isomers.

The interconversion of different topological isomers is accomplished by the transient introduction of single or double-stranded cuts into DNA; enzymes that catalyse the former reaction being classified as a type I DNA topoisomerases, the latter a type II. Some topoisomerases can supercoil DNA, some are able to relax positive and negative supercoils, others can relax positive or negative supercoils, whilst some can decatenate catenated rings or sister chromatids. No topoisomerase has been identified that is able to catalyse all of these reactions, however. Indeed, as more topoisomerases are discovered, the degree of specialisation that each of these enzymes has evolved becomes more apparent.

1.5.1 Type I topoisomerases.

1.5.1.1 Eukaryotic topoisomerase I.

Eukaryotic topoisomerase I enzymes cut one strand of a DNA duplex allowing rotation of the severed strand relative to the uncut strand and change the linking number by one. These enzymes are able to relax both negative and positive supercoiled DNA substrates.

Type I topoisomerases have been isolated from many different eukaryotic sources including humans (D'Arpa *et al.*, 1988), *Drosophila melanogaster* (Hsieh *et al.*, 1992), *S. cerevisiae* (Goto and Wang, 1985 and Thrash *et al.*, 1985), *P. falciparum* (Tosh and Kilbey, 1995), and *Caenorhabditis elegans* (Kim *et al.*, 1996). All have apparent molecular masses of around 100 kDa, but there are exceptions. Topoisomerase I activity isolated from the kinetoplastid protozoan *Trypanosoma cruzi* (Riou *et al.*, 1983) is associated with a 65 kDa monomeric protein. This is considerably smaller than most other eukaryotic topoisomerases I apart from *Vaccinia* virus, at 32 kDa.

There is a marked degree of sequence conservation over most of the protein except the N-terminal portion. A conserved seventy amino acid domain sited close to the the C-terminus has been implicated in catalysis, and mutational replacement of tyrosine 727 in *S. cerevisiae* results in an inactive enzyme suggesting that this residue is necessary for catalytic activity (Eng *et al.*, 1989).

A reaction intermediate, called the cleavable complex, can be trapped using a protein denaturant (SDS or strong alkali). Under these conditions, the enzyme becomes covalently bound to the 3' phosphoryl end of the severed DNA strand of the duplex via a specific phosphate/tyrosyl bond, leaving the 5' hydroxyl free (Champoux, 1981). This process is not dependent on the hydrolysis of ATP and no co-factor is required.

In yeast, *top I* mutants are viable, suggesting that the protein is not essential (Thrash *et al.*, 1985). However, genetic experiments with *top1* mutants have shown that the enzyme is essential for growth and development in *D. melanogaster* (Lee, 1993) and in the mouse embryo (Morham *et al.*, 1996).

Lue (1995) has crystallised a 26kDa fragment of the *S. cerevisiae* protein

(residues 135-363). Combined crystallographic and biochemical analysis of the fragment indicates that it corresponds to the region of the protein that is involved in DNA binding and catalysis. The DNA/protein interaction occurs on one surface of the fragment which has a strong electrostatic potential, implying that electrostatic forces may play a part in the interaction. Two separate regions of the fragment were found to be involved in DNA binding. It is further postulated that the residues which when mutated confer resistance to the TopoI inhibitor camptothecin (Phe293 and Gly295), map to a loop that forms part of the DNA-binding surface. Because the drug is thought to stabilise the covalent protein-DNA intermediate, it is feasible that this loop may be in the vicinity of the active site of the enzyme.

Recently, TopoI has also been shown to exhibit functions distinct from DNA relaxation. Rossi *et al.*, (1996) have reported the purification of a kinase activity which can be inhibited by camptothecin and specifically phosphorylates SR proteins involved in RNA splicing. Furthermore, Gobert *et al.*, (1996) have shown that treatment of human MCF7 breast adenocarcinoma cells with mitomycin C, a known anticancer agent, results in increased TopoI activity in parallel with an increase in p53 levels. TopoI was also found to co-immunoprecipitate p53 suggesting that the activation of TopoI may be related to an interaction between these two proteins. Purified recombinant proteins were also tested *in vitro* and p53 was again demonstrated to enhance the catalytic activity as measured by relaxation of supercoiled DNA, stabilisation of the covalent TopoI-DNA complex in the presence of camptothecin, and phosphorylation of SR protein splicing factor ASF/SF2. p53 was also found to increase the thermal stability of TopoI, protecting it from heat denaturation. These results suggest that the p53-mediated response to DNA damage may involve TopoI activation.

1.5.1.2 Bacterial topoisomerase I.

E. coli topoisomerase I was the first topoisomerase to be identified (Wang, 1971). The enzyme catalyses the relaxation of negatively supercoiled DNA. The enzyme is a 97 kDa protein which unlike eukaryotic topoisomerase I,

TopoI of *E. coli* has a requirement for exogenous magnesium as a co-factor. The DNA cleavage reaction occurs between the DNA phosphodiester bond and the active site tyrosine 319, which yields a covalent intermediate in which the tyrosine becomes linked to the 5' phosphoryl end of the cut DNA strand. Nucleophilic attack by the severed DNA strand on the phosphotyrosine linkage rejoins the DNA strand and dissociates the enzyme. The three-dimensional crystal structure of the N-terminal 67 kDa fragment of the protein has been determined (Lima *et al.*, 1994).

In *E. coli*, the competing activities of TopoI and the type II topoisomerase gyrase, act to regulate the degree of intracellular DNA supercoiling. *topA* null mutants of *E. coli* can be tolerated, however, provided compensatory mutations occur at other loci that change the degree of DNA supercoiling. One such compensatory mutation in the gene encoding DNA gyrase subunit B (*gyrB*) leads to a ten-fold reduction in enzyme activity. (McEachern and Fisher 1989).

1.5.2 Topoisomerase III.

Yeast topoisomerase III is a bacterial "TopoI" like enzyme with apparent molecular mass of 74 kDa (Wallis *et al.*, 1989). The enzyme has 21% identity to the TopoI of *E. coli*. Unlike yeast and *E. coli* topoisomerases I, yeast TopoIII is somewhat inefficient at relaxing negatively supercoiled DNA, and positively supercoiled DNA is completely refractory to it. This suggests that its biological role *in vivo* is unlikely to be related to relaxation events that occur in the cell. TopoIII can be converted into a more processive enzyme, however, and use of a 29 nucleotide long single-stranded DNA loop within a negatively supercoiled substrate DNA has demonstrated this (Kim and Wang 1992). In fact, both yeast and *E. coli* TopoIII activity can be enhanced by the presence of single-stranded regions in the DNA substrate. The minimal requirement for catalysis with the *E. coli* homologue turns out to consist of 6 bases 5' to the cleavage site and 1 base 3' to the site in single-stranded DNA. Nuclease P1 protection has shown that the enzyme binds asymmetrically, protecting 12 bases (Zhang *et al.*, 1995).

Yeast and *E. coli top3* mutants both exhibit a hyper-recombination phenotype suggesting that TopoIII may act as a suppressor of genetic recombination *in vivo*. In line with this observation, overexpression of a truncated human TopoIII (hTOP3 gene isolated by Hanai *et al.*, 1996) has been found to partially correct multiple aspects of the autosomal recessive disorder Ataxia-telangiectasia (A-T) phenotype (progressive cerebellar degeneration, immunodeficiency, accelerated aging, increased risk of cancer and genetic instability). Yeast TopoIII interacts with the putative DNA helicase SGS1 *in vivo* and *sgs1* mutants can suppress the hyper-recombination phenotype of *top3* mutants. At the same time, it has also been observed that *sgs1* mutants also exhibit a hyper-recombination phenotype. These findings suggest that TopoIII and SGS1 proteins interact to maintain genetic stability in the cell and that imbalances in either protein increase the rate of mitotic recombination. Fritz *et al.*, (1997), have also suggested that human TopoIII may act in concert with other proteins to maintain chromosome stability. SGS1 shares extensive sequence homology with BLM, the Bloom syndrome gene and WRN, the Werner syndrome gene, mutations of which cause human genetic diseases associated with hyper-recombination. It is further speculated that TopoIII/SGS1 complex may act in concert with BLM and/or WRN DNA helicases to form "reverse gyrase" like complexes *in vivo*. Wang, (1991) has also proposed that such an enzyme may be a component of a cellular complex involved in prevention of incorrectly paired DNA strands. Another interesting possibility is that TopoIII may be able to reduce recombination by preventing the formation of Z-DNA and other recombinogenic structures.

1.5.3 Reverse gyrase.

This enzyme is found in thermophilic archaeobacteria and eubacteria. In common with DNA gyrase, it has a novel intrinsic supercoiling activity but no relaxation activity, and in this respect is quite unlike any other type I topoisomerase discovered to date. This enzyme is able to catalyse the introduction of positive supercoils at high temperature, into closed circular DNA substrates at the expense of ATP. Confalonieri *et al.*, (1993)

have proposed a model for the mechanistic action of the enzyme in which supercoiling is driven by a helicase plus topoisomerase. The *Sulfolobus acidocaldarius* DSM 639 gene encodes a protein of 1247 amino acids and sequence analysis suggests the protein consists of two domains. The N-terminal domain although without similarity to other topoisomerases, contains several helicase motifs, and an ATP binding site, while the C-terminal domain of ~ 630 amino acid residues appears to be related to eubacterial topoisomerase I and also to the *S. cerevisiae* TOP3 gene. The biological function of reverse gyrase is as yet unclear.

1.5.4 Type II DNA topoisomerases.

The reactions of the type II topoisomerases are summarised in figure 3. Type II enzymes catalyse the formation of transient double-stranded breaks in a DNA duplex, a process dependent on the hydrolysis of ATP and presence of divalent cations. A second intact DNA duplex is then passed through the double-stranded gap. The process occurs by means of nucleophilic attack on a 5' staggered pair of phosphodiester bonds four base pairs apart (Morrison and Cozzarelli 1979). The 5'-phosphoryl ends of the duplex become covalently linked to the phenolic oxygens of the tyrosine groups present on each subunit of the enzyme. This forms the basis of a DNA gate through which the second duplex can be transported. Following transportation, a transesterification reaction occurs; this is mediated by the reaction of the two 3'-hydroxyl groups present on the severed DNA ends and the phosphotyrosine bonds to the protein. This reaction seals the broken duplex without damaging the DNA (Roca and Wang 1994). Eukaryotic topoisomerase II relaxes both negative and positive supercoiled DNA.

1.5.4.1 Eukaryotic topoisomerase II

The catalytic cycle of eukaryotic topoisomerase II has been elucidated by Osheroff *et al.*, (1991) and comprises the six following steps; (Step 1) involves substrate recognition and binding, and is determined by topological structure and nucleotide sequence. In humans and chicken the sequence is reported to be;

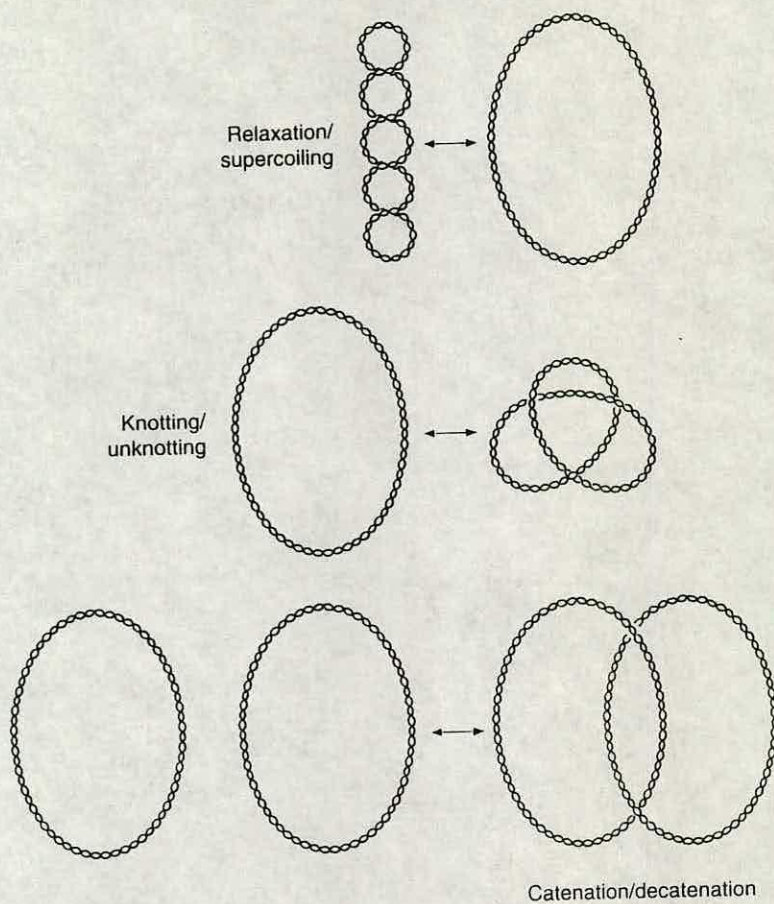
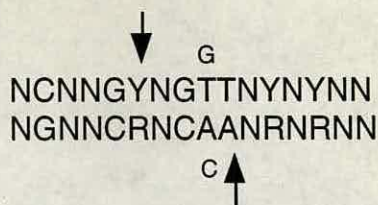


Figure 3.
The reactions of type II topoisomerases.



Where Y = pyrimidines, R = purines and N = any complementary base (G and C are alternative recognition sites).

TopoII can discriminate between different topological structures and interacts preferentially with supercoiled substrates. This is important because it enables the enzyme to distinguish between the supercoiled substrate and the products of its own reaction.

Step 2: Following substrate recognition, topoisomerase II establishes a rapid double-stranded DNA cleavage/religation equilibrium, which favours religation. This process requires the presence of a divalent cation, which is normally magnesium *in vivo*, but calcium can substitute *in vitro*. Transesterification from the phosphodiester DNA backbone to the catalytic tyrosine, results in a covalent bond between each subunit of the protein. The cut introduced into the DNA substrate is staggered with a 4-base 5' overhang and evidence suggests that the cuts are introduced sequentially. This step is the target of several classes of antineoplastic drugs (eg. amsacrine and etoposide) which stabilise the topoisomerase II/DNA cleavage complex, by decreasing the apparent first order rate constant for religation.

During the third step, following strand breakage, strand passage occurs, a process which depends on ATP binding and results in the relaxation of two negative supercoils. Whilst in the fourth step, following strand passage, topoisomerase II establishes another cleavage/religation equilibrium. This step has not been studied in detail due to the difficulty involved in isolating the intermediate, however this step is also a target for certain classes of inhibitor which act by stabilising the post-strand

passage enzyme/DNA complex, inhibiting the religation event as in Step 2 although their effects appear to be less dramatic than in the pre-strand passage step.

In Step 5 bound ATP is hydrolysed to ADP and orthophosphate, whilst in Step 6 enzyme turnover occurs after the protein has dissociated from the DNA substrate. Under optimal conditions the enzyme is highly processive remaining bound to the DNA until all supercoiling has been removed.

Berger *et al.*, (1996), have crystallised a 92 kDa fragment of the yeast protein (residues 410-1202) which comprises the central domain and part of the C-terminal domain (shown in Fig. 4 a). The structure is heart shaped with a central hole. The 92 kDa fragment can cleave DNA but cannot execute duplex transport because it lacks the ATPase domain. Using information already obtained on the crystal structure of the ATPase domain from *E. coli* GyrB, Berger *et al.*, have proposed a molecular model of the enzyme as an ATP-modulated clamp with two sets of jaws at opposite ends, connected by multiple joints (Fig. 4 b).

Figure 4 (a).

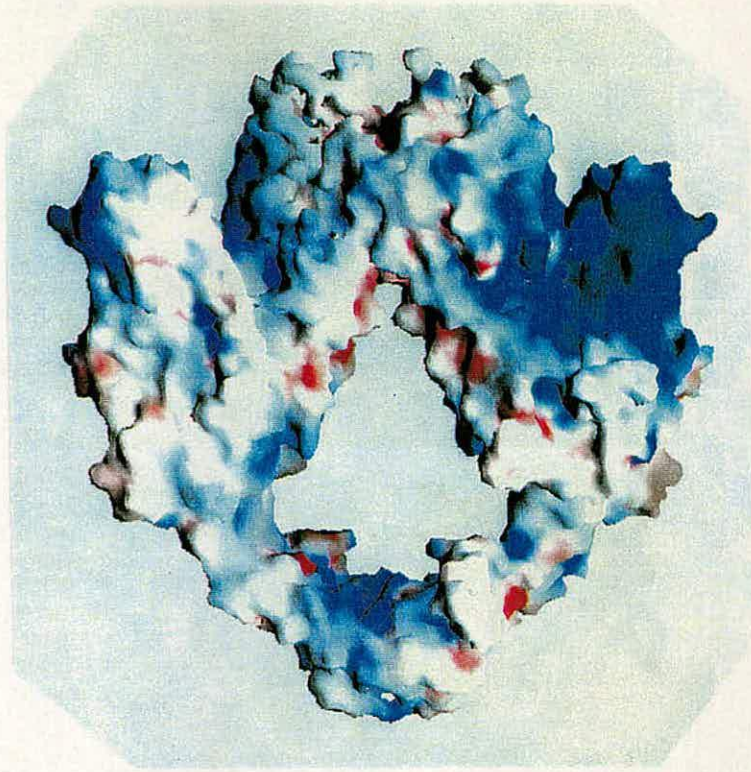
Representation of the yeast DNA topoisomerase II (410-1202) dimer. Two crescent-shaped monomers form a pair to make a heart shaped dimer with a large central hole. The A' subfragment of each monomer contains residues 682-1178 and the B' subfragment residues 420-633. Subfragments belonging to one subunit are shaded more darkly than their counterparts.

Figure 4 (b).

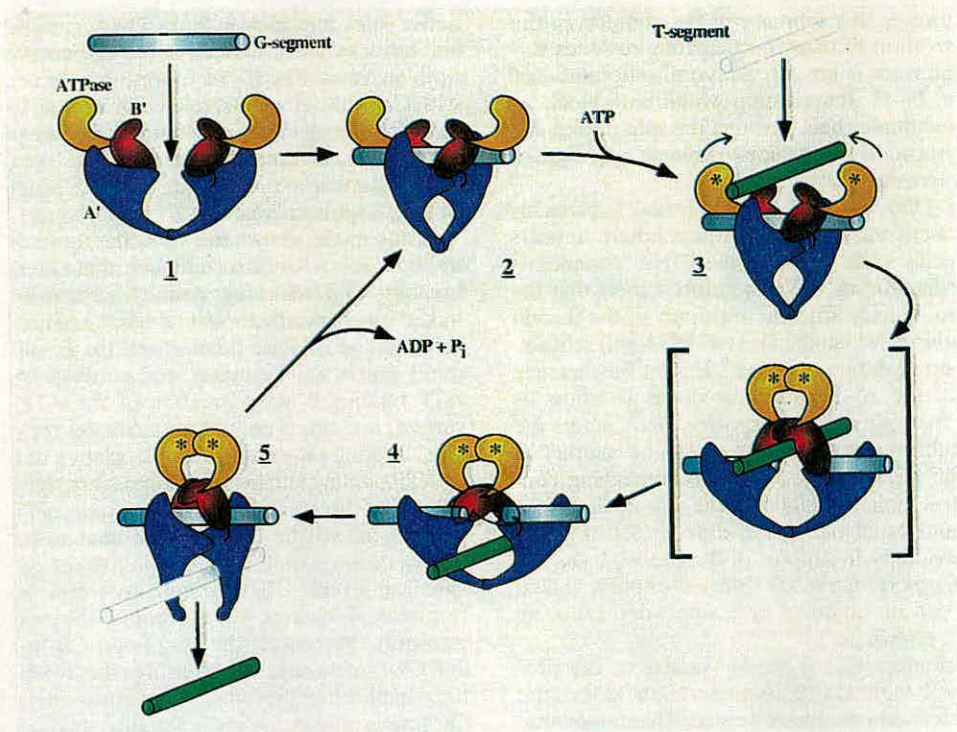
The molecular model proposed for the catalytic reaction of topoisomerase II. The G-segment DNA (containing the DNA gate) is coloured grey and the transported T-segment is green. (1) enzyme binds the G-segment DNA which induces the conformational change shown in (2). ATP binding (represented by asterisks) and T-segment binding (3) induce additional conformational changes in which the G-segment is broken by the A' subfragments as they separate from each other. Concomitantly, the ATPase domains dimerise, and the T-segment is transported through the break and into the central hole (4). The B' subfragment in front is uncoloured in (4) to permit visualisation of the DNA behind it. For clarity, the DNA transport step is shown to proceed through a hypothetical intermediate (brackets). Following transport, the G-segment is resealed and the T-segment released from the enzyme through the opening of the dimer interface between A' subfragments (5). The interface between the two A' subfragments again dimerises, and ATP is hydrolysed and released to regenerate the starting state (2).

Figures and text copied and adapted from Berger *et al.*, (1996).

a



b



The sequences encoding several topoisomerases II have been determined, including the yeasts *S. cerevisiae* (Giaever *et al.*, 1986), *S. pombe* (Uemura *et al.*, 1986) and *Candida albicans* (Keller *et al.*, 1997), *D. melanogaster* (Wyckoff *et al.*, 1989), *Trypanosoma brucei* (Strauss and Wang 1990) and *P. falciparum* (Cheesman *et al.*, 1994). The apparent molecular masses of each subunit range in size between 155 to 170 kDa which comprise holoenzymes of 310-340 kDa.

Eukaryotic topoisomerases II share extensive homology over the N-terminal region of the protein. This is especially so for the residues that appear to have known functions. The N-terminal domain is homologous to the B subunit of the bacterial gyrase homologue. It contains a highly conserved glycine which is involved in ATP binding (residue 147 in PfTopoII). The structure of this region in the gyrase protein has been defined by x-ray diffraction (Wigley *et al.*, 1991). The central region of the protein corresponds to the gyrase A subunit and contains the tyrosine residue (residue 829 in PfTopoII) that is involved in strand-breakage and religation. This region also contains the highly conserved motif *LIMTDQD*, which has been used as a means to isolate the gene in many species, including *P. falciparum* where the leucine residue is replaced with a methionine (*MIMTDQD*).

Although the C-terminal domain appears not to have any known catalytic function and is remarkably species divergent, it is retained in many phylogenetically distinct eukaryotic organisms. Thus it appears most likely that this region performs some important function for the enzyme. Indeed, a nuclear localisation signal is reported to be located within the most distal sixty amino acids in *Drosophila* (Greenwood-Crenshaw and Hsieh 1993) and a putative leucine zipper motif has also been identified in many species which may function in subunit dimerisation (Caron and Wang 1994).

Related to this, phosphorylation of the C-terminal domain has been proposed as a post-translational modification governing enzyme activity. The *D. melanogaster* homologue is phosphorylated *in vitro* by casein kinase II. In this case, phosphorylation led to a 3-fold stimulation of DNA relaxation activity compared with the unphosphorylated form. These

effects could be reversed by alkaline phosphatase (Ackerman *et al.*, 1985). Yeast topoisomerase II has also been shown to be a phosphoprotein and it has been proposed that the existence of casein kinase II sites in the C-terminal domain form part of the mechanism by which control of enzyme activity is achieved (Cardenas *et al.*, 1992).

Recently, the C-terminal domain of TopoII (*S. cerevisiae*) has been assigned another role. Watt *et al.*, (1995) used a two-hybrid cloning strategy to isolate a protein called Sgs1 p, which is structurally related to the *E. coli* Rec Q helicase protein. Sgs1 p interacts with a short region of the C-terminal domain of TopoII that has been implicated in dimerisation of the TopoII protein. Strains lacking a functional *SGS1* gene show a reduced fidelity of both mitotic and meiotic chromosome segregation, which leads to a diminished capacity to undergo cell division. Based on these findings, Watt *et al.*, (1995) have proposed a model in which Sgs1 p and TopoII interact during the final stages of DNA replication to achieve chromosome segregation.

Two distinct DNA topoisomerases II (α and β) have been isolated from humans which share 72% identity. The 170 kDa α isoform (Tsai-Pflugfelder *et al.*, 1988) is present in proliferating cells, is localised in the nucleoplasm and has a dual structural/enzymatic role. This isoform shares 65% sequence similarity with the *P. falciparum* homologue.

The 180 kDa human isoform is encoded by a different genetic locus to the α isoform, and has been designated topoisomerase β (Jenkins *et al.*, 1992 and Austin *et al.*, 1993); it is reported to be present in cells which have reached the plateau growth phase, and is localised to the nucleolus (Negri *et al.*, 1992). There is speculation that localisation to the nucleolar remnant may indicate that it could represent a structural element for the spatial organisation and regulation of transcription of ribosomal genes (Zini *et al.*, 1994).

It has been suggested that the different isoforms may have arisen by a gene duplication event which occurred relatively recently. Although the β form is larger by around 100 amino acid residues at the carboxy terminus, the functional significance of this is unknown. One distinct difference between the *P.falciparum* protein and all other eukaryotic

topoisomerases II is the presence of two unique adjacent polyasparagine rich tracks situated distal of the N-terminal domain. No functional role has been assigned to these inserts.

Topoisomerase II is a multifunctional enzyme which plays several roles in DNA metabolism. One of these roles relates to the segregation of chromosomes following DNA replication during mitosis and meiosis in yeast (DiNardo *et al.*, 1984 and Holm *et al.*, 1985). *S. cerevisiae top2* mutants have been shown to accumulate catenated dimers derived from newly replicated circular plasmids (DiNardo *et al.*, 1984). Failure to segregate these catenated DNA intermediates ultimately results in cell death when the cell attempts to divide at mitosis (Holm *et al.*, 1985). Similar findings have been reported with living *Drosophila* cells, following immunodepletion of TopoII, daughter chromosomes fail to separate at anaphase (Buchenau *et al.*, 1993). Thus it seems most likely that TopoII activity is required as a decatenase, at least for circular or large genomes. But which topoisomerase is responsible for providing the swivelase activity required for the early stages of DNA replication at the replication fork? Work conducted by Goto and Wang 1984, Uemura and Yanagida 1984 and Brill *et al.*, 1987, has indicated that although either topoisomerase can provide swivelase activity during the early stages of DNA replication, topoisomerase I is the most likely candidate. This is supported by studies with SV40 where TopoII inhibitors slowed only the last 5% of genome replication (Snapka *et al.*, 1988).

The use of topoisomerase inhibitors has yielded valuable information. In mammalian cells, the topoisomerase I inhibitor camptothecin has been shown to stop DNA replication almost immediately, lending support to the idea that TopoI is probably the major swivelase. The relatively new drug ICRF-193, has also been used to gain insight into the early stages of DNA replication. It is likely to be more informative than some of the earlier inhibitor studies using drugs like VP-16 and VP-26, which work by stabilising the cleavable complex thereby yielding unreliable results due to drug induced DNA damage. This damage causes blocks in chain extension at an early stage in during replication. ICRF-193 causes cell death during the G2/M phase and thus mimics the phenotype of yeast *top2* mutants,

unlike VP-16 and VP-26 which on the other hand cause cell death during S phase. The outcome of studies using ICRF-193 further support the idea that TopoII is not required during the early stages of DNA replication, but is needed for the resolution of late replication intermediates (Ishimi *et al.*, 1992 and Clarke *et al.*, 1993).

Several lines of evidence now exist to indicate that TopoII appears unlikely play a direct role in transcription. Shaak *et al.*, (1990) have pointed out that levels of TopoII in terminally differentiated and quiescent cells are very low, even though these cells are transcriptionally active. In addition, VP-26 does not reduce transcription of HeLa heat shock genes or adenoviral genes, whilst use of camptothecin led to a reduction of adenovirus and HeLa cell mRNA.

Although genetic studies with yeast have demonstrated that topoisomerase I is dispensable for viability, it is also known that a lack of this protein leads to a reduction in growth rate and a decrease in large RNA transcripts (Brill *et al.*, 1987).

TopoII is a major non-histone protein component of the metaphase scaffold and acts as a scaffold protein possibly joining the A/T rich DNA sequences found at the base of chromosome loops, the scaffold attachment regions (SAR), to the nuclear scaffold (reviewed in Gasser and Laemmli 1987). Supporting this, consensus cleavage sites for TopoII are found in SAR sequences (Udvardy *et al.*, 1985).

Apart from its known catalytic and structural roles, TopoII also plays a role in chromosome condensation. In cultured mammalian cells, treatment of G-2 phase cells with the TopoII inhibitor VM-26 prevents chromosome condensation and entry into mitosis (Lock and Ross 1990) but it is unclear whether lack of a functional TopoII prevents entry into mitosis or whether the block is a result of DNA damage caused by the drug. More convincing studies have been conducted by Adachi *et al.*, (1991), who has demonstrated that adding back TopoII to immunodepleted cells restores their ability to condense chromosomes effectively.

1.5.5 Type II bacterial DNA topoisomerases.

1.5.5.1 DNA gyrase and topoisomerase IV.

DNA gyrase is a type II enzyme first identified in 1976 by Gellert *et al.*, that can introduce and relax negative supercoiling in steps of two. This process requires ATP as an energy cofactor. The protein is encoded by the *gyr A* and *gyr B* genes; the active enzyme is a heterotetramer composed of two A and two B protein subunits (A₂/B₂ configuration) which bind DNA and wrap about 120 base pairs around the protein core. The individual subunits are 97 kDa for the A subunit and 90 kDa for the B subunit. The gyrase B protein contains the active site tyrosine residue involved in the strand-breakage religation reaction, (Tyr 122) whilst the gyrase A protein contains the ATPase domain of the protein. The crystal structure of the N-terminal fragment of the B protein has been elucidated by Wigley *et al.*, 1991.

For many years gyrase was believed to be responsible for unlinking newly replicated daughter chromosomes, but it is now known that topoisomerase IV fulfils this role *in vivo*. TopoIV is encoded by the *parC* and *parE* genes. In common with other type II enzymes except gyrase, TopoIV has no intrinsic supercoiling activity, but does possess a potent decatenase activity. Mutations in the genes encoding both gyrase and TopoIV are conditionally lethal. *parC* and *parE* mutations results in a terminal partitioning defect whereas mutations in the *gyrA* and *gyrB* genes lead to an “immediate-stop” DNA replication phenotype.

Bacterial gyrase is the target of two main classes of inhibitors, but their bacteriocidal action is mediated by two different mechanisms; the quinolones, in particular the highly potent fluoroquinolones (eg. ciprofloxacin) work by interfering with the rejoining of double-stranded breaks in the DNA, whilst the coumarins (eg. novobiocin) inhibit the ATPase activity of the enzyme. Quinolones also target TopoIV.

1.6 Topoisomerase inhibitors.

DNA topoisomerases I and II are the cellular targets of a group of diverse drugs in clinical use as anti-tumor agents. Although the drugs are structurally dissimilar, their mechanism of action involves either

inhibition of the DNA strand-breakage/reunion reaction (Type I or II enzymes) or inhibition of ATP hydrolysis (Type II enzymes).

1.6.1. Eukaryotic topoisomerase I poisons.

Camptothecin and related compounds.

Camptothecin is a plant alkaloid isolated from *Camptotheca acuminata*. Camptothecin does not appear to react with DNA or RNA polymerases or with DNA itself, nor does it react directly with topoisomerase II, but in the presence of topoisomerase I, large amounts of single-stranded DNA breaks accumulate (Hsiang *et al.*, 1985). The cytotoxic effects are thought to result from the accumulation of enzyme-DNA linkages when cleavable complexes are stabilised and trapped by the drug.

However, despite the potentially promising antitumor properties exhibited by camptothecin, early clinical trials during the 1970's showed not only that the drug was lacking activity, but also that it was highly toxic. At this stage however, the target of camptothecin was unknown. Chemical modifications of the basic compound have produced new analogues in the late 1980's which appear to be not only highly active, but also less toxic. Irinotecan, for example, is a semisynthetic analogue of camptothecin whose metabolite SN-38 is 100-fold more active. This drug has recently been registered in Japan, USA and most European countries, where its use is indicated for colorectal cancer which is unresponsive to most other cytotoxic treatment regimes. Topotecan is another semisynthetic water soluble TopoI inhibitor based on camptothecin which has recently been registered for use against ovarian cancer. 9-Aminocamptothecin, 9-Nitrocamptothecin, Lurtotecan (GI 147211), DX 8951 and Ho 33342 are TopoI inhibitors currently undergoing clinical development.

TopoI inhibitors are arguably the most important class of cytotoxic agents developed over the last decade.

1.6.2 Topoisomerase II poisons and inhibitors.

It has been established that the cellular level of topoisomerase II increases when quiescent cells are stimulated to proliferate, and decreases when cells

are induced to differentiate. This may explain the sensitivity of proliferating and tumor cells to topoisomerase II poisons. The specificity of different chemical compounds for topoisomerases has enabled synthetic organic chemists to design novel drugs which are selective against the topoisomerases of pathogenic species including bacteria, fungi and protozoan parasites.

Topoisomerase II poisons and inhibitors can be grouped according to whether they act as DNA intercalators or non-intercalators. A brief discussion of some of the drugs that poison or inhibit topoisomerase II follows.

1.6.2.1 m-AMSA (amsacrine).

Amsacrine is a clinically active antileukemia agent first reported in 1971 as a potentially useful therapeutic agent. Although amsacrine is reported to be active against myelogenous leukaemia, toxicity problems have limited its clinical usefulness.

Studies have shown that the drug binds to DNA *in vitro* with the acridine moiety responsible for the intercalation between and parallel to the base pairs. This causes the double helix to extend and become distorted while the anilino moiety projects into the minor groove where it interacts with the enzyme (Marshall and Ralph 1985). It has also been shown that the drug stimulates the formation of enzyme-DNA complexes (Nelson *et al.*, 1984).

The nature of the substituents on the anilino moiety dramatically alters the antitumor activity of 9-anilinoacridines, possibly by altering the contact with the enzyme. The structural isomer *o*-AMSA is known to be inactive, here the 3' methoxy group is in the 5' position.

Many other intercalative drugs exist that target topoisomerase II by forming non-productive drug-enzyme-DNA ternary complexes eg. the ellipticines, actinomycin D, doxorubicin and daunomycin.

Another DNA intercalator, ethidium bromide has also been investigated and was found not to stimulate topoisomerase II-mediated DNA cleavage demonstrating that intercalation *per se* is not sufficient to elicit this response (Pommier *et al.*, 1983). Other intercalative antitumor drugs

include daunomycin, doxorubicin, mitoxantrone, and actinomycin D.

1.6.2.2 Non-intercalating antineoplastic drugs.

The epipodophyllotoxins are semisynthetic derivatives of the natural product podophyllotoxin. Two members of this group are etoposide (VP-16) and teniposide (VM-26). These are clinically active against Kaposi's sarcoma, testicular carcinoma, leukemia and small cell lung cancer. Neither of the drugs bind isolated DNA but are capable of stabilising the eukaryotic topoisomerase II/ DNA cleavable complex, and are reported to be active at late S and G2 phases of the cell cycle (Krishan *et al.*, 1985). In clinical trials, teniposide has been found to be 10-fold more active than etoposide, but this increased activity may be a function of cellular uptake (Sinha, 1993).

Human leukaemia cell lines selected for resistance to teniposide have been isolated (Bugg *et al.*, 1991). The basis of resistance appears to be related to a single mutation in the topoisomerase II gene, where arginine 449 has changed to glutamine. Nuclear extracts from these cell lines have decreased levels of topoisomerase II catalytic activity and decreased capacity to form drug-stabilised covalent complexes. The ATP concentration required for equivalent activity in a DNA unknotting assay was found to be 2 to 8-fold higher in nuclear extracts derived from these drug-resistant cell lines compared to parental lines, indicating that the alteration in ATP binding observed may be related to drug resistance.

The difficulties involved in performing genetic analysis in mammalian cell lines has complicated the determination of whether mutations occurring in the topoisomerase II gene are responsible for drug resistance. Nitiss *et al.*, (1994) have attempted to address this problem by constructing a yeast topoisomerase II gene with an equivalent mutation to that found in the human leukemia cell line discussed above. In this case, mutation of lysine 439, the equivalent amino acid in the yeast protein to either glutamine or glutamic acid was found to confer resistance to both etoposide and amsacrine.

1.6.2.3 ICRF-193.

ICRF-193 is a member of the bisdioxopiperazine class of drugs and inhibits topoisomerase II by a novel mechanism. In the presence of ATP the enzyme appears to become trapped in what is thought to be the closed clamp form of the enzyme and is thus unable to cleave DNA (Roca *et al.*, 1994).

1.6.2.4 Dual Inhibitors.

A small number of inhibitors exist which are known to target both TopoI and TopoII; these include saintopin, actinomycin D and intolincine. Leteurtre *et al.*, (1994), studied TopoI and TopoII cleavable complexes in the presence of saintopin and found the same preference for a guanidine base located 3' to the topoisomerase-induced break at position + 1. Furthermore, a camptothecin-resistant topoisomerase I with Asn 722 mutated to Ser next to the catalytic site tyrosine (position 723) was found to be cross resistant to saintopin, indicating that both camptothecin and saintopin interact with TopoI near the active site tyrosine.

1.6.2.5 9-anilinoacridines (mechanism of action unknown).

Widespread recognition of human topoisomerase II as the target for chemotherapeutic agents has highlighted the potential benefit of targeting the protein in other pathogenic organisms, including protozoan parasites. The effects of certain 9-anilinoacridine derivatives have now been investigated for a number of parasites including *Leishmania*, *Trypanosoma* and *Plasmodium*.

Leishmaniasis (Kala-azar) is a severe tropical disease for which relatively few clinically effective drugs exist that do not cause severe side effects. Around 12 million people are infected each year, following transmission by female sandflies, hence the impetus for new drug development. Werbovets (1994) has tested a range of anilinoacridines using the parent compound m-AMSA as a standard, and has obtained encouraging results with some of these compounds. At cytotoxic concentrations some of these compounds were found to generate cleavable complex formation with whole cell parasite extracts, suggesting the the cellular target may be

TopoII.

A range of 9-anilinoacridine compounds, have also been tested against the multi-drug resistant KI isolate of *P. falciparum* (Chavalitsheewinkoon *et al.*, 1993, and Gamage *et al.*, 1994) and the outcome of these studies were discussed earlier.

1.7 Summary and scope of this thesis.

Malaria continues to pose a severe world health problem, with over 40% of the world's population now at risk from the disease. The emergence of multi-drug resistant parasites which are increasingly refractory to most of the chemotherapeutic agents commonly employed for treatment is serious cause for concern. Hence there is an urgent need to identify novel antimalarial drug targets within the parasite. One such potential target in the parasite is topoisomerase II, which in other organisms is the target of effective antineoplastic and antimicrobial drugs.

This project arose naturally from earlier work, where the gene encoding PfTopoII was isolated and characterised as a preliminary step in the evaluation of the enzyme as a target for antimalarial drugs. Purification of the protein directly from the parasite was not undertaken due to the difficulties involved in culturing the very large number of blood stage parasites required to yield reasonable quantities of protein for biochemical analysis. As an alternative, the heterologous expression of the protein was attempted as a means to provide sufficient recombinant protein for drug screening and analysis. This thesis describes our attempts to achieve heterologous expression of PfTopoII, and attempts to understand why achieving it was so difficult. In addition, data is presented on the stage-specific expression pattern for PfTopoII during the parasite's developmental stages that are most closely associated with the clinical symptoms of the disease.

Chapter 2

2.1 Materials

2.1.1 Equipment

High speed centrifugation was conducted with either a Sorvall RC5-B high speed centrifuge (DuPont Instruments) or Sorvall OTB50-B ultracentrifuge (DuPont Instruments). Benchtop centrifugation was carried out using a Heraeus Biofuge 13 (Eppendorf, Germany) or a Jouan CR322 (Saint-Herbain, France). PCR was carried out using a GeneE thermal cycler (Techne Instruments). Hybridisations were done in HB-1 or HB-1D heated cabinets (Techne Instruments). Plasticware and cell culture materials were supplied by Beckton Dickinson Labware, UK.

2.1.2 Chemicals

Unless specified otherwise, all chemicals were supplied by Sigma Chemical Co. Ltd, UK. Cell culture materials were supplied by Gibco Life Sciences, UK. Radionucleotides were supplied by Amersham International, UK., or ICN Flow, UK. Solvents were supplied by FSA Laboratory Supplies, UK.

2.1.3 Restriction and modifying enzymes.

Unless otherwise stated, restriction endonucleases and buffers were supplied by Boehringer Mannheim UK (Diagnostics and Biochemicals) Ltd., UK. Modifying enzymes were supplied by Gibco-BRL, UK or Promega UK.

2.1.4 Autoradiography

KODAK X-OMAT AR and X-OMAT LS autoradiography film was supplied by IBI Molecular Biology Products, UK.

2.1.5 Photography

UV-irradiated ethidium bromide-stained gels were photographed with either HP5 film, ILFORD Ltd., UK. or Mitsubishi Video Processor to heat sensitive film.

2.1.6 Microbiology

2.1.6.1 Table 1. Bacterial strains.

Strain	Genotype	Reference
DH5α	F', <i>endA1, recA1, hsd</i> R17(<i>r_k⁻, m_k⁺</i>), <i>sup</i> E44, <i>thi</i> -1, <i>gyrA</i> 96, <i>relA1</i> , ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lac</i> ZYA- <i>argF</i>), U169, <i>deoR</i> .	Hanahan (1983)
BL21 (DE3)	F ⁻ , <i>hsdS_B</i> , Δ (<i>lac</i> U169) <i>gal</i> (<i>lc</i> 1857 <i>ind1</i> <i>Sam7</i> <i>nin</i> 5 <i>lacUV5-T7 gene1</i>), (<i>r_B⁻, m_B⁻</i>), <i>ompT, lon</i> . [pLysS]	Studier and Moffat (1986) Studier (1991)
INVαF'	F', <i>endA1, recA1, hsd</i> R17 (<i>r_k⁻, m_k⁺</i>), <i>sup</i> E44, <i>thi</i> -1, <i>gyrA</i> 96, <i>relA1</i> , ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lac</i> ZYA- <i>argF</i>), U169, λ^- .	Hanahan (1983) (Ultracompetent cells supplied by Invitrogen)

2.1.6.2 Table 2. Yeast strains.

Strain	Genotype	Reference
JEL 1	<i>MATa, ura3-52 ,leu2, trp1, prbl-1122, pep 4-3, Δhis3, :: GAL10-GAL4</i> (a protease deficient strain)	Dr. C. Austin (pers comm.).
SD117	<i>MATa, ade2, leu2, his7, top2-1, trp1, ura3-52</i>	Brill <i>et al.</i> , (1987).
SD119	<i>MATa, top1-1, top2-1, trp1, ura3-52</i>	Brill <i>et al.</i> , (1987).
JCW26	<i>top2-4, ura3-52, leu2, trp1</i>	Hsiang <i>et al.</i> , (1985).
JCW28	<i>top1, top2-4, ura3,-52 leu2, trp1</i>	Hsiang <i>et al.</i> , (1985).

2.1.6.3 Table 3. Insect cells and baculoviruses.

<i>Spodoptera frugiperda</i> fall armyworm	Described by O'Reilly <i>et al.</i> , 1992.
<i>Autographa californica</i> nuclear polyhedrosis virus (AcMNPV)	Described by O'Reilly <i>et al.</i> , 1992.

2.1.6.4 Table 4. Plasmids.

pUBS1	Gowman <i>et al.</i> , 1991.
pUC19	Yanisch-Perron <i>et al.</i> , 1985.
pGEX2-T	Smith & Johnson, 1988.
pRSETB	Schoepfer, 1993.
pLysS	Studier, 1991.
YEplac33	Gietz & Sugino, 1988.
YEplac181	Gietz & Sugino, 1988.
YEplac195	Gietz & Sugino, 1988.
YEpWob6	Wasserman <i>et al.</i> , 1993.
p218 (a pBluescript derivative)	Gift - Dr. Caroline Austin.
pVL1393	Gruenwald & Heitz, (1993).
pAcSG His	Supplied by PharMingen.
pCR TM II	Mead <i>et al.</i> , 1991.

2.2. General stock solutions and media.

Amino Acids (yeast experiments).

<u>Constituent</u>	<u>final mg/l</u>	<u>stock per 100ml</u>	<u>ml per 100 ml</u>
Adenine sulphate	20	120 mg	1.66
L-histidine-HCl	20	240 mg	0.83
L-leucine	30	360 mg	0.83
L-tryptophan	20	240 mg	0.83
Uracil	20	240 mg	0.83

Denaturation Solution.

1.5M sodium chloride and 0.5M sodium hydroxide.

Glass beads.

Glass beads were prepared by soaking in concentrated nitric acid for 1 hour, washing extensively in sdH₂O and baking until dry.

20 % Lactate pH 5.7 solution.

Lactic acid 85% was obtained from Sigma Chemicals. The 85% solution was diluted in sdH₂O in a beaker placed over ice in a fume hood to give a final concentration of around 30%. The solution was placed on a magnetic stirrer with a large stirrer bar and KOH pellets added until the solution reached a pH of 5.7. sdH₂O was added to bring the solution to 20% and then autoclaved in 50 ml aliquots.

Luria- Bertani medium (LB, supplied by ICMB media service).

1% bacto-tryptone; 0.5% Bacto-yeast extract; 1% sodium chloride adjusted to pH 7.2, using 1M sodium hydroxide.

LB agar (supplied by ICMB media service).

LB supplemented with 1.5% agar.

Minimal medium.

80 ml Spitzizen salts (5 X - supplied by ICMB media service).

10 ml glucose (20% w/v); 1mg/ml vitamin B1 and 320 ml sdH₂O.

Neutralising Solution.

1M ammonium acetate; 0.02M sodium hydroxide.

Oligonucleotide Hybridisation Buffer.

2XSSC; 0.2% SDS; 0.1% sodium pyrophosphate and 500µg/ml heparin.

Phosphate Buffered Saline (PBS).

137mM sodium chloride; 2.7mM potassium chloride; 4.3mM disodium hydrogen orthophosphate and 1.4mM potassium dihydrogen orthophosphate.

Pre-Hybridisation Buffer.

0.25M sodium phosphate, pH 7.2; 7% SDS.

Protein loading buffer - reducing (2 X)

62.5mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS (w/v); 5% β -Mercaptoethanol.

Rifampicin.

1mg/ml in dimethylformamide.

SOC medium.

2% tryptone; 0.5% yeast extract; 10mM sodium chloride; 2.5mM potassium chloride; 10mM magnesium chloride; 10mM magnesium sulphate and 20mM glucose.

Standard Saline Citrate (SSC) 20x stock solution.

3M sodium chloride; 100mM trisodium citrate, pH 7.0.

Tris-Borate-EDTA (TBE) 10x stock solution.

0.9M Tris borate; 20mM EDTA, pH 8.0.

Tris- EDTA (TE).

10mM Tris-HCl, pH8.0; 1mM EDTA, pH 7.5 or pH 8.0.

Tris- EDTA-lithium acetate (10 X stock).

100mM Tris-HCl, pH 7.5; 10mM EDTA and 1M lithium acetate, pH 7.5.

Tris- EDTA-lithium acetate-40% PEG.

10mM Tris-HCl, pH 7.5; 1mM EDTA; 100mM lithium acetate, pH 7.5 and 40% (w/v) PEG 4000.

Tris- EDTA- Saline (TES).

20mM Tris-HCl, pH 7.5; 10mM EDTA and 100mM sodium chloride.

Tris-Saline.

10mM Tris-HCl, pH 7.4; 150 mM NaCl.

Tris-Saline-NP40.

10mM Tris-HCl, pH 7.4; 150 mM NaCl and 0.05% NP40 (v/v).

Tris-Saline-5% Marvel.

10mM Tris-HCl, pH 7.4; 150 mM NaCl and 5% powdered milk (w/v).

Yeast minimal medium + agar.

For 100 ml of medium.

0.66g yeast nitrogen base without amino acids (DIFCO); 2% glucose; 2% Bacto-agar (DIFCO), and appropriate amino acid.

Yeast minimal medium.

For 100 ml of medium

0.66g yeast nitrogen base without amino acids (DIFCO); 2% glucose and appropriate amino acid.

Yeast minimal medium with glycerol and lactate.

For 100 ml of medium

0.66g yeast nitrogen base without amino acids (DIFCO); 2% glycerol; 2% lactate and appropriate amino acid.

Yeast cell lysis Buffer 1.

1 X TEE (50mM Tris-HCl, pH 7.7; 1mM EDTA; 1mM EGTA) containing 10% glycerol, 25mM sodium fluoride, 1mM sodium bisulphite, 1mM PMSF, 1mM DTT, 1mM benzamide, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A.

Unless otherwise stated the methods described in the following text were based on those found in either Sambrook *et al.* (1989), Harlow and Lane (1988) or O'Reilly *et al.* (1992).

2.3 DNA Methods

2.3.1 Isolation of DNA.

Plasmid DNA was prepared from *Escherichia coli* using either the QIAGEN Plasmid Midi Kit, or the WizardTMPlus Minipreps DNA purification system described below.

2.3.1.1 Midipreparation of plasmid DNA.

100ml of LB medium supplemented with 100µg/ml ampicillin was inoculated with a single colony and grown at 37°C overnight with shaking. 25ml of the culture was centrifuged (2500g for 10 minutes). The supernatant was discarded and the bacterial pellet resuspended in 4 ml of buffer P1 (50mM Tris-HCl, pH 8.0; 10mM EDTA; 100µg/ml RNase A). 4 ml of cell lysis buffer P2 (200 mM NaOH; 1% SDS) was added and the suspension incubated for 5 minutes at room temperature. The solution was neutralised by mixing with 4 ml of buffer P3 (3 M potassium acetate, pH 5.5) and incubated for 15 minutes on ice. The sample was centrifuged (20,000g, 4°C, 30 minutes), and the supernatant removed promptly. The sample was re-centrifuged for 15 minutes to remove any remaining particulate matter. A QIAGEN tip 100 was equilibrated with 4 ml of buffer QBT (750mM NaCl; 50mM MOPS, pH 7.0; 15% ethanol; 0.15% Triton X-100) by gravity flow. The cleared supernatant was applied to the column by gravity flow. The column was washed twice with 10 ml of buffer QC (1M NaCl; 50mM MOPS, pH 7.0; 15 % ethanol). DNA was eluted with 5ml of buffer QF (1.25M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol), precipitated with 0.7 volumes of room temperature isopropanol, then centrifuged (15,000g, 4°C, 30 minutes). The pellet was washed with 2 ml of 70% ethanol, air dried briefly, and dissolved in 200µl TE (10mM Tris-HCl, pH 8.0; 1mM EDTA). The DNA was analysed by UV spectrophotometry.

2.3.1.2 Isolation of small scale plasmid DNA.

Wizard™ Plus Minipreps protocol.

5 ml of LB medium supplemented with 100µg/ml ampicillin was inoculated with a single colony and grown at 37°C overnight with shaking. 1-3 ml of the culture was centrifuged (10,000g, for 2 minutes). The bacterial pellet was resuspended in 200µl of 50mM Tris-HCl, pH 7.5; 10mM EDTA and 100 µg/ml RNase A. 200µl of lysis solution (0.2M NaOH; 1% SDS) was added and the solution mixed by inverting four times. Alkalinity was neutralised by addition of 1.32M potassium acetate, and the solution centrifuged (10,000g for 5 minutes). The cleared supernatant was transferred to a clean tube, 1ml of the purification resin was added, and

the solution mixed and applied to the miniprep column using a 5ml syringe. The column was washed with a solution containing 80mM potassium acetate; 8.3mM Tris-HCl, pH 7.5; 40 μ M EDTA and 55% ethanol. The column was dried by centrifugation (10,000g for 30 seconds) and the DNA eluted in 50 μ l of TE pH8 (10,000g for 20 seconds). The DNA was analysed by agarose gel electrophoresis or UV spectrophotometry.

2.3.1.3 Isolation of plasmid DNA from *Saccharomyces cerevisiae*.

This protocol is based on the method of Hoffman and Winston (1987). A single colony harbouring a recombinant *E. coli*/yeast shuttle-plasmid was picked into 5 ml of selective culture medium and grown overnight at 30°C with agitation. The cells were harvested by microcentrifugation for 5 seconds. The supernatant was discarded and the pellet resuspended in the residual liquid by vortexing briefly. 0.2 ml of 2% Triton X-100; 1% SDS; 100mM NaCl; and 10mM Tris-HCl, pH 8.0 were added along with 0.2 ml phenol-chloroform-isoamylalcohol (25:24:1, v/v/v). 0.3 g of 0.4mm glass beads were added and the solution vortexed for 2 minutes prior to pelleting by microcentrifugation for 5 seconds. 1-5 μ l of the aqueous layer was used for transformation of *E. coli* cells (2.3.6.2).

2.3.1.4 Isolation of total DNA from *Spodoptera frugiperda* insect cells.

1 X 10⁶ non-infected insect cells, and recombinant or wild type infected insect cells that were still attached to tissue culture dishes, were incubated with 5ml of lysis buffer (30mM Tris-HCl, pH 7.5; 10mM magnesium acetate; 1% NP40 v/v), for 10 minutes on ice. The lysate was transferred to a 15ml conical tube (Sterilin), the solution vortexed for 20 seconds, and centrifuged (1000g for five minutes at 4°C). The supernatant was discarded and the pellet gently resuspended in 5 ml of ice-cold phosphate buffered saline (PBS) pH 6.2, and re-centrifuged. The pellet was resuspended in 200 μ l of extraction buffer (10mM Tris-HCl, pH 8.0; 5mM EDTA; 0.5% SDS). Proteinase K was added to a final concentration of 1 mg/ml and the solution incubated at 37°C with gentle mixing for 4 hours. The sample was extracted by addition of 200 μ l of phenol/chloroform/IAA, pH 8.0 (prepared and used as described in sections 2.3.1.6.1 and 2.3.1.6.2) with

gentle mixing, before centrifugation in a benchtop centrifuge at top speed for 1 minute. The upper aqueous phase was collected, avoiding the interface between the organic and aqueous phases. This procedure was repeated twice. An equal volume of chloroform was added to each sample, and the extraction process repeated a further two times. DNA was precipitated as described in section 2.3.1.7.

2.3.1.5 UV spectrophotometry.

The concentration and purity of nucleic acids was quantified using UV spectrophotometry. DNA or RNA samples were diluted 1 in 200 in sterile water and analysed on the Perkin Elmer 115 spectrophotometer set up to scan the range 200-300nm. The concentration of the sample was deduced from the formula:

DNA concentration = absorption at 260nm (A_{260}) X 50 X dilution factor.

RNA concentration = absorption at 260nm (A_{280}) X40 X dilution factor.

The relative purity of the sample was determined by calculating the ratio A_{260}/A_{280} , where a value of 1.8 or greater indicates that the sample is free of protein contamination.

2.3.1.6 Phenol/chloroform extraction of samples.

2.3.1.6.1 Preparation of phenol/chloroform.

Phenol was pH buffered prior to use to make it slightly alkaline. A small spatula tip full of 8-hydroxyquinine was allowed to dissolve in 5ml of water saturated phenol. 2ml of 1M Tris-HCl, pH 8.0 was added and the solution mixed, allowed to settle, and centrifuged for 2 minutes at 1000g. The aqueous phase was removed and 2ml of TE pH 8.0 was added. The solution was mixed, allowed to settle, then centrifuged as before. 5ml of chloroform, 0.2ml isoamyl alcohol, and 2ml TE pH8.0 were added, and the solution was stored at 4°C for up to three months.

2.3.1.6.2 Phenol chloroform extraction.

DNA was extracted by adding an equal volume of phenol/chloroform solution, mixed vigorously by hand, then spun in a microfuge at high speed for 1 minute. The upper aqueous phase containing the DNA was

transferred to a clean tube and the sample was re-extracted again, up to three times, until the interface between the organic and aqueous phases was clean. The aqueous phase was then extracted against chloroform to remove any traces of phenol, prior to ethanol precipitation.

2.3.1.7 Ethanol precipitation.

0.1 volume of 3M sodium acetate, pH 5.2 and 2 volumes of ice-cold ethanol were added to the DNA solution. The sample was incubated at -70°C for at least 15 minutes to promote precipitation of the nucleic acid. The sample was centrifuged at high speed in a microfuge for 30 minutes and the pellet washed with 70% ethanol, followed by one wash with 100% ethanol. The pellet was air dried and resuspended in TE pH 8.0. The DNA was analysed by agarose gel electrophoresis and UV spectrophotometry (2.3.1.5).

2.3.2 Endonuclease restriction digests of DNA.

Restriction digests of DNA were conducted for 2-3 hours at 37°C for all restriction endonucleases except *Sma*I digests which were conducted at 30°C. 3-10 units of commercial restriction endonucleases were used to restrict 1µg of DNA in the presence of the appropriate buffer, supplied as a 10X stock concentration and diluted to a final concentration of 1X in a total volume of 20µl to 50µl. For single restriction digests, Table 5 was consulted for the appropriate buffer, but for multiple restriction digests involving enzymes that are not active together in the same buffer, "One-Phor-All" buffer (Pharmacia) was used instead (100mM Tris-acetate; 100mM magnesium acetate and 500mM potassium acetate) as an alternative to heat inactivation of enzyme and ethanol precipitation between each individual digest.

Table 5. Composition of Boehringer Mannheim buffers (mM)

A	Buffer				Composition
	B	L	M	H	
33	-	-	-	-	Tris acetate
-	10	10	10	50	Tris-HCl
10	-	-	-	-	Magnesium acetate
-	5	10	10	10	Magnesium chloride
-	100	-	50	100	Potassium acetate
-	-	1	1	1	Dithioerythritol
0.5	-	-	-	-	Dithiothreitol
-	1	-	-	-	β -mercaptoethanol
7.9	8.0	7.5	7.5	7.5	pH at 37°C

* *Kpn*I restriction digests were supplemented with BSA at 100 μ g/ml.

2.3.3 Agarose gel electrophoresis.

2.3.3.1 Casting agarose gels.

Multi-purpose agarose was supplied by Boehringer Mannheim.

Table 3. Relationship between concentration of agarose and separation of linear DNA molecules. Sambrook *et al.*, (1989).

Percentage agarose	Separation of linear DNA molecules
0.7	0.8-10 kbp
0.9	0.5-7 kbp
1.2	0.4-6 kbp
1.5	0.2-3 kbp

Multi purpose agarose was dissolved by heating to boiling point in 0.5 or 1X TBE buffer (2.2). The solution was cooled to approximately 55°C and ethidium bromide was added to a final concentration of 0.2 μ g/ml. The molten gel was poured into the appropriate size casting tray and allowed to solidify at room temperature.

2.3.3.2 Running agarose gels.

The solidified gel was immersed in 0.5 X or 1 X TBE buffer. Linear DNA size standards or samples were run with 1 X sample buffer (30% glycerol; 0.25% bromophenol blue; 0.25% xylene cyanol; 100mM EDTA, pH8.0). Samples were electrophoresed at 15Vcm⁻¹ until sufficient separation of the DNA fragments was observed, as determined by short wave UV transillumination of the gel.

2.3.3.3 Photodocumentation of agarose gels.

The image was recorded on heat sensitive film or a negative image was produced using FP5 film (Kodak). Gels to be Southern blotted were photographed with a ruler adjacent to the left hand side of the gel for alignment purposes.

2.3.3.4 DNA size standards.

The DNA markers routinely used for size determination were λ DNA restricted with *Eco*RI and *Hind*III (Promega). These markers produce DNA fragments of approximately 23.15, 9.42, 6.56, 4.38, 2.32, 2.02 and 0.56kbp.

2.3.3.5 Electroelution of DNA fragments from agarose gels.

The DNA fragment of interest was excised from the agarose gel using a hand held short wave UV light source. Dialysis tubing was prepared by boiling in 2% sodium hydrogen carbonate, 1mM EDTA for 10 minutes with gentle intermittent stirring. The membrane was extensively washed with sterile distilled water, and reboiled in 1mM EDTA for a further 10 minutes before being washed in water, immersed in 70% ethanol and kept at 4°C for long term storage. Prior to use, the membrane was washed in sterile distilled water.

The DNA agarose slice was inserted into 2-3" of membrane that was clipped at one end. 400 μ l-1ml of 0.5X or 1X TBE buffer was added to the partially sealed membrane, air was expelled and the membrane was clip sealed at the other end. The "bag" was placed into a horizontal

electrophoresis tank and a current of 100V was applied for approximately 30 minutes. The current was reversed for the final 30 seconds to disperse any DNA that may have adhered to the inside of the membrane. Elution was checked using short wave UV transillumination. The DNA was precipitated with ethanol, dissolved in T.E. and its concentration estimated by agarose gel electrophoresis.

2.3.4 Southern Blotting agarose gels.

2.3.4.1 Dry Southern blotting.

After photography, the agarose gel was immersed in denaturing solution (section 2.2) for 15 minutes. When large DNA fragments of greater than 15kb were present, the gel was depurinated using several volumes of 0.2M HCl for 10 minutes, prior to rinsing with distilled water. The gel was then neutralised (section 2.2) for 30 minutes. Hybond N (Amersham) or Genescreen Plus (DuPont) membrane was cut to exactly the same size as the gel, and the right hand corner of both the gel and membrane was removed to aid future orientation. The membrane was wetted with neutralisation solution and placed on top of the gel slowly to avoid the introduction of air bubbles. Three pieces of blotting paper (Whatman) were wetted with neutralisation solution and placed on top of the gel/membrane sandwich. On top of this, two dry pieces of blotting paper and a stack of paper hand-towels were assembled. A large lead weight was placed on top of the assembly, and the transfer of nucleic acid allowed to proceed for a minimum of 2 hours to overnight, according to the size of the DNA fragments. The membrane was removed and rinsed briefly in 2 X SSC (2.2) and allowed to air dry. Short wave UV light was used to crosslink the DNA to the membrane (Hybond N membranes). For Genescreen Plus membranes crosslinking is not necessary so the membrane was washed in 2 X SSC then transferred into prehybridisation buffer (section 2.2).

2.3.4.2 Wet Southern Blotting.

Wet Southern transfer was used in cases where the DNA was present in relatively small quantities (<100ng). Depurination, denaturation and

neutralisation of DNA was conducted as described previously (section 2.3.4.1). The prepared gel was placed on a glass plate covered with a blotting paper "wick" underneath it, above a reservoir containing 10 X SSC into which the "wick" was immersed. The gel was surrounded with Saran wrap to prevent the 10 X SSC solution bypassing the gel and soaking the paper towels. The gel was covered in Genescreen Plus (DuPont) cut to exact size. Five sheets of dry blotting paper and a 5" stack of paper towels were placed on top of the membrane. A glass plate was placed on top of the gel assembly, and on top of this a heavy lead weight. Transfer was left to proceed overnight. Next morning the blot was disassembled and the membrane rinsed in 2 X SSC prior to prehybridisation and probing (section 2.3.4.3).

2.3.4.3 Prehybridisation and hybridisation of radiolabelled probes.

The same solution was used for prehybridisation and hybridisation of membranes, but the composition of the solution varied according to whether the membrane was to be exposed to end-labelled oligonucleotides (6 X SSC, 500 μ g/ml heparin; 0.1% SDS; 0.1% sodium pyrophosphate) or random-labelled probes (0.25M sodium phosphate; 7% SDS). The membrane was transferred to a glass hybridisation tube, and incubated overnight with 25ml of prehybridisation solution. The following day the solution was replaced, the probe added and incubated for a minimum of 6 hours for end-labelled probes, and overnight for random-labelled probes.

2.3.4.4 5' end labelling of oligonucleotides.

End-labelled probes were used when the target DNA was present in reasonable quantity (>25ng). Prehybridisation and hybridisation were done at 37°C for oligonucleotides of <20 bases in length. Oligonucleotides of >20 bases were hybridised at temperatures compatible with their melting temperature. The end labelling reaction contained the following; 80ng of oligonucleotide (without 5' phosphate), 2 μ l of 10 X Reaction buffer (700mM Tris-HCl, pH 7.6; 100mM magnesium chloride; 50mM DTT), 12 μ l sdH₂O, 1 μ l (γ ³²P ATP) [10mCi/ml-1] and 1 μ l T4 polynucleotide kinase (10



Units/ μ l).

The reaction was incubated at 37°C for 1-2 hours. At this stage the probe was added to the prehybridised membrane.

2.3.4.5 Random labelled probes.

Random labelled probes were made using the Prime-It™II Random Primer Labelling Kit (Stratagene). All materials were supplied in this commercially available kit, with the exception of the T7 DNA polymerase which was supplied by Promega. The reaction was prepared as follows; 25ng of double-stranded DNA in $x \mu$ l; 10 μ l of random labelled primers (9-mers at 8ng/ μ l) and sdH_2O to 25 μ l. The solution was boiled for 5 minutes and then snapped cooled on ice. The following were then added; 10 μ l of 5 X random label buffer (dCTP), containing 250mM Tris-HCl, pH 8.0; 100mM magnesium chloride; 20mM β -mercaptoethanol; and 0.5mM each of dGTP, dATP, and dTTP; 3 μ l [α ^{32}P] dCTP (10mCi/ml⁻¹) and 0.5 μ l T7 DNA polymerase (8.5 units/ μ l). The reaction was incubated at 37°C for 10 minutes and 2 μ l of 0.5M EDTA was added to stop the reaction.

Removal of unincorporated dNTPs was achieved by use of a NucTrap™ Probe Purification Column (Stratagene). 70 μ l of TES (100mM sodium chloride; 20mM Tris-HCl, pH 7.5; 10mM EDTA) were pushed slowly through the column using a 10ml syringe. 20 μ l of TES were added to the reaction and the solution was pushed through the column. An additional 70 μ l of TES was pushed through the column and the random-labelled probe collected in a tube. The solution was boiled for 10 minutes to separate the DNA strands, prior to adding to the prehybridised membrane. Random-labelled probes were hybridised to membranes at 65°C.

2.3.4.6 Washing of membranes and signal detection.

2.3.4.6.1 Removal of unbound 5' end labelled oligonucleotide probes.

The probe was transferred from the hybridisation tube and stored shielded at -20°C to decay. The membrane was then washed with 25ml of 6 X SSC/0.1% SDS, twice for 15 minutes. The membrane was monitored with

a β -geiger counter to determine whether any additional washes were required prior to exposure to film. Up to 2 further washes in 6 X SSC would be done if necessary. Washed membranes were placed into a plastic bag and heat sealed and a fluorescent strip (Stratagene) was positioned next to the membrane for orientation purposes. Membranes were exposed to pre-flashed film and stored at -70°C in a cassette with intensifying screens. Variable periods of exposure were used according to the strength of the signal estimated from the β -Geiger counter (cps). The film was developed using a X-OGRAPH X1 (IBI Ltd.) automatic X-ray film developer.

2.3.4.6.2 Removal of unbound random-labelled probe.

The probe was transferred from the tube and stored (2.3.4.6.1). To remove unbound probe, the membrane was washed twice with 2 X SSC/0.1% SDS for 15 minutes, followed by 1 X SSC/0.1% SDS twice for 15 minutes. The membrane was monitored with a β -geiger counter to determine whether a more stringent wash with 0.2 X SSC/0.1% SDS for 15 minutes was required. The membrane was treated as described in 2.3.4.6.1.

2.3.4.7 Membrane stripping.

2.3.4.7.1 5' end-labelled oligonucleotide probes.

The membrane was stripped by placing it in a hybridisation tube, at 65°C containing 0.1 X SSC, 0.1% SDS for 1 hour. The membrane was exposed to film overnight to ensure that no signal was detected.

2.3.4.7.2 Random-labelled probes.

Random-labelled probes were stripped from membranes by placing in a hybridisation tube at 65°C , containing 0.4M sodium hydroxide. After 30 minutes the solution was replaced with neutralisation solution (0.1 X SSC; 0.1% SDS; 200mM Tris-HCl, pH 7.5) for 30 minutes at 65°C . The membrane was exposed overnight to ensure that no signal was detected.

2.3.5 Ligations.

2.3.5.1 End filling 3' recessed termini.

The 5' to 3' polymerase activity of Klenow (Promega) was used to generate blunt termini from 3' recessed termini. 1 μ l of 1mM dCTP, dGTP, dATP and dTTP were added to 0.2 to 0.5 μ g of DNA dissolved in 20 μ l of sdH₂O. Magnesium chloride was added to a final concentration of 5mM, along with 1 unit of Klenow. The reaction was allowed to proceed for 15 minutes at room temperature, after which the enzyme was heat inactivated at 75°C for 10 minutes. The mixture was cooled slowly to room temperature prior to ligation.

2.3.5.2 Phosphatase treatment of restricted vector.

The 5' phosphate of a restricted vector was removed with calf intestinal alkaline phosphatase (CIP) to prevent self-ligation. The restriction endonuclease was removed by phenol/chloroform extraction followed by ethanol precipitation. The DNA was resuspended in 20 μ l of 1 X CIP buffer (50mM Tris-HCl, pH 8.5; 1mM EDTA; 1mM magnesium chloride; 1mM zinc chloride). 1unit of CIP was added and the reaction incubated at 37°C for 30 minutes. 1/10 volume of 200mM EGTA, pH 8.2 was added and the reaction was heat treated to inactivate the CIP at 75°C for 15 minutes. The reaction was phenol/chloroform extracted and ethanol precipitated before ligation.

2.3.5.3 Ligation reactions.

The vector and insert were restricted as required and their ends were modified as necessary. Typically, 40ng of vector was ligated to 40ng of insert, at a range of different vector to insert ratios. For example, ratios of 1:1 and 1:3 were routinely used. Both vector and insert DNA were added to 1 X ligation buffer (66mM Tris-HCl, pH 7.6; 6.6mM magnesium chloride; 10mM DTT; 66 μ M ATP) with 1 unit of T4 DNA ligase (Gibco BRL). Ligations were incubated overnight at 15°C.

2.2.5.4 Ligation of linkers to blunt-ended DNA fragments.

Blunt-ended DNA was used as substrate for the enzymatic addition of

phosphorylated linkers containing a restriction endonuclease site. 0.1 μ g of DNA and 2 μ g of phosphorylated *Kpn*I linkers (Gibco BRL) were assembled in an Eppendorf in a total volume of 7 μ l. 2 μ l of 10 X ligation buffer was added (2.2.5.3) and the reaction catalysed by addition of 1 unit of T4 DNA ligase (Gibco BRL). Ligations were incubated overnight at 15°C.

2.3.5.5 PCR product cloning.

PCR products were subcloned using the commercially available TATM Cloning Kit (Invitrogen). This kit works by exploiting the fact that *Taq* polymerase has a non-template dependent activity which adds a single deoxyadenosine to the 3' end of PCR products. The linear vector used in this kit has single 3' deoxythymidine residues. This allows the PCR product to ligate efficiently to the vector.

1 μ l of fresh (less than one day old) PCR product was added to 2 μ l of TA vector pCRTM II (25ng/ μ l), 1 μ l of 10 X ligation buffer (60mM Tris-HCl, pH 7.5; 60mM magnesium chloride; 50mM NaCl; 1mg/ml bovine serum albumin; 70mM β -mercaptoethanol; 1mM ATP; 20mM DTT; 10mM spermidine), 5 μ l sdH₂O, and 4 units of T4 DNA ligase. The reaction was incubated at 15°C overnight.

2 μ l of 0.5M β -mercaptoethanol was added to One ShotTM INV α F' competent cells after defrosting on ice. 2 μ l of the ligation mix was added with gentle mixing to the cells. The transformation mix was heat shocked at 42°C for 30 seconds and placed on ice for 2 minutes. 450 μ l of room temperature SOC was added and the transformation mix incubated for 1 hour at 37°C on a rotary wheel. The cells were set on ice for 2 minutes, prior to spreading aseptically in 50 μ l and 200 μ l aliquots onto LB plates supplemented with 100 μ g/ml ampicillin and 0.004% X-Gal. The plates were allowed to dry, inverted and incubated at 37°C for 18 hours. To promote full colour development, the plates were transferred to 4°C for 2 hours. White colonies were then tested for the presence of PCR inserts by restriction digest.

2.3.6 Preparation of competent *Escherichia coli* cells and transformation.

2.3.6.1 Preparation of competent *Escherichia coli* cells.

A single colony was inoculated into 5ml of LB medium and incubated with shaking overnight at 37°C. Next morning, 2ml of the culture was added to 100ml of fresh LB medium, the cells incubated with shaking at 37°C for 2 hours, and cooled on ice for 10 minutes. The cells were collected by centrifugation (1500g, 10 minutes, 4°C). The pellet was gently resuspended in 20ml of 100mM calcium chloride and incubated on ice for a minimum of 2 hours or overnight. The cells were collected by centrifugation as above and resuspended in 1/20 volume of 100mM calcium chloride. Any unused cells were stored at -70°C in 100mM calcium chloride, 14% glycerol in 200µl aliquots for periods of up to 6 months.

2.3.6.2 Transformation of competent *E. coli* cells.

Competent cells were transferred from the -70°C freezer and allowed to defrost slowly on ice. Typically between 1-5µl of a ligation was incubated with 200µl of competent cells on ice for 30 minutes. The solution was mixed very gently with the end of a pipette tip to avoid mechanical damage to the cells. The transformation mix was then transferred to a 42°C waterbath to heat shock the cells (45 seconds for DH5α, or 1 minute for BL21 cells). The mixture was immediately returned to ice for 2 minutes before adding SOC or LB medium and incubating with agitation at 37°C for 60 minutes. The transformed cells were plated out in 200µl aliquots onto selective medium and incubated in an inverted position at 37°C overnight.

2.3.6.3 Preparation of competent *Saccharomyces cerevisiae* cells.

This method is based on the protocol of Gietz *et al.*, (1992). A single yeast colony was inoculated into liquid YDPA medium and grown on a shaking incubator overnight at 30°C to 1-2 X 10⁷ cells/ml. The culture was then diluted ten-fold into fresh YDPA and regrown to 1 X 10⁷ cells/ml. Cells were harvested (1500g for 5 minutes) and washed in 1 ml of sterile distilled water before transferring to a 1.5 ml tube and pelleting at top

speed for 1 minute in a bench top microfuge. The cells were washed in 1 ml of TE/lithium acetate made fresh from 10 X stocks of TE, pH 7.5, and lithium acetate (2.2), resuspended at 2×10^9 cells/ml in 1 X TE/lithium acetate. At this stage the cells were determined competent and were transformed immediately.

2.3.6.4 Transformation of competent *Saccharomyces cerevisiae* cells.

50 μ l of the competent cell suspension was mixed with 1 μ g of transforming DNA and 50 μ l of single-stranded salmon sperm carrier DNA and 300 μ l of sterile 40% PEG 4000/lithium acetate/TE solution (2.2). The mixture was incubated at 30°C with agitation for 30 minutes. The cells were heat shocked for 15 minutes in a water bath at 42°C, then harvested in a bench top microfuge at top speed for 5 seconds. The pellet was resuspended in 1 ml of 1 X TE pH 7.5 and spread onto selective medium plates which were inverted once dry and transferred to a 30°C incubator. The plates were kept at 30°C until colonies were visible, a process which took up to 5 days.

2.3.6.5 Preparation of selective medium plates.

2.3.6.5.1 Ampicillin supplemented LB plates.

100 mg/ml stock solution of ampicillin was prepared in sdH₂O and used at a final concentration of 100 μ g/ml in LB medium. This was used as selective medium for all experiments with DH5 α or BL21 cells.

2.3.6.5.2 Yeast minimal plates.

Yeast minimal selective medium containing 2% glucose, glycerol/lactate, or galactose was prepared according to section 2.2 but omitting the amino acid required for selection.

2.3.6.5.3 Chloramphenicol/ampicillin supplemented plates.

20mg/ml stock solution of chloramphenicol was prepared in 100% ethanol and used at a final concentration of 20 μ g/ml, with ampicillin at a final concentration of 100 μ g/ml in LB medium. This was used as selective media for all experiments using BL21 (DE3) [pLysS] cells.

2.3.7 Colony screening.

2.3.7.1 Blue/white colour selection.

The insertion of a DNA fragment into the multiple cloning site of a vector containing the *lacZ* gene which facilitates blue/white colour selection was tested by plating onto medium containing 200mM IPTG and 0.004% X-GAL in DMF. Following incubation overnight at 37°C, plates were transferred to 4°C to allow full colour development to occur. White and light blue colonies were picked for further analysis.

2.3.7.2 Patching white colonies.

This method was used to test white colonies identified by blue/white selection for the presence of inserts. Individual white colonies were picked using a sterile toothpick and scratched onto a Hybond N (Amersham) membrane which had been stamped with a 100 square matrix and placed onto a selective media plate. The same colony was also scratched onto a selective media plate without a membrane, but with a similar matrix placed underneath the plate to enable identical colonies to be identified. Both plates were incubated overnight at 37°C. The colonies which had grown on the membrane were lysed by treating with 2 X SSC/5% SDS for 2 minutes, and the DNA fixed to the membrane by microwaving on full power for 1 minute. The membrane was then ready for prehybridisation.

2.3.7.3 Colony lifts.

This protocol is based on the method of Buluwela *et al.* (1989). Hybond N membranes were cut to the same size as the petri plate and placed onto the surface. The membrane was marked for orientation purposes by puncturing with a needle through to the medium underneath. The membrane was carefully removed and soaked colony side up in 2 X SSC/5% SDS as described in section 2.3.7.2.

2.3.8 Polymerase chain reaction (PCR).

2.3.8.1 Standard PCR reaction.

PCR reactions were based on the methods of (Saiki *et al.*, 1988 and Innis *et*

al., 1990) but were modified to take into account the relatively high A+T content of the parasite template DNA. PCR reactions were assembled on ice and care was taken to ensure that cross contamination of tubes did not occur.

100ng template DNA	x µl
10 X PCR buffer (500 mM KCl; 100 mM Tris-HCl, pH 9.0; 1% Triton X-100)	10 . 0µl
25mM Magnesium chloride	6 . 0µl
Forward oligonucleotide (20 ng/µl)	10 . 0µl
Reverse oligonucleotide (20 ng/µl)	10 . 0µl
100mM dNTP's (25 mM each of dATP, dTTP, dCTP, dGTP)	0 . 7µl
<i>Taq</i> (<i>Thermus aquaticus</i>) DNA polymerase (5U/µl)	0 . 5µl
sdH ₂ O to a total of 100 . 0µl	

Taq DNA polymerase and reaction buffer were supplied by Promega UK. The PCR reactions were overlaid with 50µl of mineral oil before being placed onto the thermocycler. A typical programme for the thermocycler for a pair of 17-mer oligonucleotide primers using a genomic or plasmid DNA template to amplify a kilobase pair product is described below. Modifications were made to this standard protocol for the amplification of longer DNA fragments or for the amplification of DNA fragments that were to be used for expression.

Denaturation	Annealing	Polymerisation
(1) 95°C for 5 minutes	37°C for 1 minute	70°C for 2 minutes
(2) 93°C for 3 minutes	37°C for 1 minute	70°C for 2 minutes
(3) 93°C for 3 minutes	40°C for 1 minute	70°C for 2 minutes
(4)		70°C for 5 minutes

(1) and (2) were set up to run for 1 cycle each, (3) was set up for between 10 to 28 cycles and (4) was set up for 1 cycle only.

The maximum ramp rate (1° C sec⁻¹) between each step was used in all PCR reactions. 1/10 volume of the PCR product was analysed by agarose gel electrophoresis.

2.3.8.2 Reverse-transcriptase PCR (RT-PCR).

Following completion of the first strand cDNA synthesis (2.4.4) a PCR was used to amplify the cDNA samples according to the First Strand cDNA Synthesis Kit protocol (Pharmacia Biotech). The primers that were used for this reaction were a sense strand PftOP2 gene specific primer (719S) and the NotI-d(T)₁₈ primer that was used to prime the reverse transcriptase reaction.

The entire reverse transcription reaction was used in the PCR which contained the following components; 45mM Tris-HCl, pH 8.3; 68mM KCl; 15mM DTT; 9mM magnesium chloride; 0.08mg/ml BSA and 1.8 mM each of dATP, dTTP, dCTP and dGTP. 0.2µg of 719S and NotI-d(T)₁₈ primers were added, together with 2.5 units of *Taq* polymerase. The standard PCR parameters described in section 2.3.8.1 were used with 30 cycles of amplification.

1µl of total DNase I treated RNA was used in a separate PCR, with same primers. This PCR provided a control for non-specific binding of the primers, in the event that DNase I treatment of the RNA samples used for RT-PCR had not worked.

2.3.9 DNA sequencing and analysis.

2.3.9.1 Manual sequencing of double-stranded DNA templates.

DNA sequencing was carried using the dideoxy-chain termination method described by Sanger *et al.*, (1977). The commercially available Sequenase^{RVII} kit (USB) was used throughout. Oligonucleotide primers were supplied by the OSWEL DNA Service, Southampton, UK.

To 4µg of double-stranded DNA, 200 mM NaOH; 2 mM EDTA, pH 8.0, and sdH₂O to a total of 30µl were added. The DNA solution was allowed to denature at 37°C for 30 minutes, before ethanol precipitation. The pellet was resuspended in 7µl of sdH₂O and kept on ice ready for the annealing reaction. 1µl of the appropriate oligonucleotide (20ng/µl) and 2µl of reaction buffer (200mM Tris-HCl, pH 7.5; 100mM magnesium chloride; 250mM sodium chloride) was added to the DNA. The reaction was placed in a large beaker containing water at 65°C and allowed to cool slowly to 30°C over a period of about 1 hour, at which point the annealing reaction

was complete.

The labelling reaction was carried out as follows; 10 μ l of the annealed DNA, 2 μ l of dGTP labelling mix (1.5 μ M of dGTP; dTTP; and dCTP) 1 μ l of DTT, 0.5 μ l of [α^{35} S] dATP (10mCi/ml⁻¹) and 2 μ l of Sequenase^RDNA polymerase (1.6 U/ μ l) were assembled and incubated at room temperature for 5 minutes before 3.5 μ l aliquots were removed and added to 2.5 μ l aliquots of the termination mixes (ddG, ddC, ddA and ddT) prewarmed to 37°C. Each termination mix consisted of the following; 80 μ M dGTP, dCTP, dATP and dTTP, 50mM sodium chloride and 8 μ M of the appropriate dideoxynucleoside triphosphate (ddGTP, ddCTP, ddATP and ddTTP). The termination mixes were incubated at 37°C for 5 minutes and the reactions stopped by the addition of loading buffer (95% formamide; 20mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol) prior to freezing at -20°C. Reaction mixes were stable for up to three days.

2.3.9.2 Sequencing gel electrophoresis.

Sequencing reactions were run on 6% polyacrylamide gels (20:1 acrylamide:bisacrylamide) containing 7M urea in 1 X TBE available commercially from Scotlab, Scotland. 40ml of polyacrylamide mix was polymerised by the addition of 240 μ l of a 10% ammonium persulphate solution prepared in water, and 24 μ l of TEMED (BRL). The gel was cast between a pair of plates 380mm by 170mm separated by plastic spacers (0.3mm). A "sharks tooth" comb was used to prepare wells at the top of the gel to facilitate loading of the samples.

Up to 5 μ l of the sequencing reaction was boiled for 2 minutes, snap cooled on ice for 2 minutes, and loaded onto the gel. Electrophoresis was carried out at 50 W and monitored by the downward movement of the two blue tracking dyes. After an appropriate period of time the electrophoresis was stopped and the plates were carefully separated. The gel was fixed by placing the plate with the gel on it into a solution of 10% glacial acetic acid, 12% methanol for up to 30 minutes. The gel was then gently lifted out of the solution and laid onto Whatman 3MM paper before drying under vacuum at 80°C. Sequencing gels were usually exposed to film overnight at room temperature.

2.3.9.3 Sequence analysis.

Storage, manipulation and comparisons of sequencing data were made using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, versions, 6,7,8 and 9 (Devereux *et al.*, 1984).

2.3.9.4 Automatic sequencing of double-stranded DNA templates.

The ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used for sequencing reactions that were to be run and analysed by the ABI 377 automated sequencer (Perkin Elmer Applied Biosystems). Oligonucleotide primers were supplied by the OSWEL DNA Service, Southampton, UK.

0.5µg of double stranded plasmid DNA, 3.2 pmole of primer, 8µl of Terminator Ready Reaction Mix (supplied in the Kit) and sdH₂O to a final volume of 20µl were added to a 0.5ml tube. The reaction was overlaid with 40µl of mineral oil before being placed onto the thermal cycler.

Thermal cycling was conducted as follows;

Step 1) 96°C for 30 sec, 50°C for 15 seconds, 60°C for 4 minutes.

Step 2) repeat step 1 for 25 cycles. The maximum ramp rate (1°C sec⁻¹) between each step was used in all reactions.

Mineral oil was removed and the completed reaction products purified by addition of 2µl of 3M sodium acetate, pH 4.6 and 50µl of 95% ethanol to precipitate the DNA. Reactions were incubated on ice for 10 minutes before centrifugation to pellet the DNA, using a benchtop microfuge at top speed for 30 minutes. The pellet was washed once with 70% ethanol, and once with 100% ethanol before air drying. Reactions were stored at -20°C for up to two days before being run on the automated sequencing machine by Nicola Preston.

2.3.9.5 Analysis of DNA sequences prepared by the automatic sequencer.

Sequencing data were downloaded onto a floppy disc and the files analysed using the ABI Prism Sequence™Navigator Programme version 1.0.1 (Perkin Elmer).

2.3.10 Generation of recombinant baculoviruses by co-transfection.

2.3.10.1 BaculoGold™ Transfection Kit (PharMingen).

The method of Gruenwald and Heitz (1993) was used for co-transfection. 2µg of recombinant plasmid DNA prepared by the QIAGEN Plasmid Midi Kit was used for each co-transfection. *Spodoptera frugiperda* (Sf9 cells) were seeded at 2×10^6 onto 60 mm round tissue culture plates. The cells were left to attach for 30 minutes at room temperature whilst the co-transfection reagents were assembled. 0.5µg of linearised BaculoGold™ DNA and 2µg of recombinant baculovirus transfer vector were mixed in an Eppendorf and allowed to sit for 5 minutes at room temperature before adding Buffer B (1ml of 125mM Hepes, pH 7.1; 125mM CaCl₂; 140mM NaCl). The medium was removed and replaced with Buffer A (Graces's Medium supplemented with 10% fetal bovine serum). The DNA and transfection solution were added dropwise to the plate and rocked gently back and forth to mix the solutions.

50µl of wild type virus *Autographa californica* nuclear polyhedrosis virus (AcNPV) were added to an Sf9 plate seeded at 2×10^6 to act as a control. A third plate was also seeded at 2×10^6 cells, but no viruses were added. All three plates were incubated at 27°C for 4 hours before removing the medium and replacing with fresh TC-100 (insect cell medium) supplemented with 10% fetal calf serum (Gibco). The plates were returned to the 27°C incubator for approximately 4 days, at which stage signs of viral infection should be apparent. The supernatant was collected from the co-transfection plate by centrifugation (1000g, for 5 minutes) and stored at 4°C in the dark until required.

2.3.10.2 Generation of pure viral stocks.

60mm cell culture dishes were seeded with 2×10^6 Sf9 cells which were incubated at room temperature for 30 minutes to allow the cells to attach to the plate. Recombinant viruses or wild type virus were serially diluted to 10^{-5} - 10^{-8} in insect cell medium. Following attachment of the Sf9 cells to each dish, the medium was aspirated and replaced with 0.5ml of the serially diluted viruses. Dishes were incubated with gentle rocking for 1 hour at room temperature. 4ml of a solution containing 25ml Graces

Insect cell medium supplemented with 20% FBS (Gibco), 12.5ml of ssdH₂O, and 4% agarose gel (Gibco) maintained at 37°C was quickly added to each dish. The solution was allowed to solidify at room temperature before incubating at 27°C for 4-6 days. At this stage the plaquing overlay was stained with 0.5ml of Neutral Red dye (Gibco) for 30 minutes followed by three 30 minute washes with TC-100 insect medium, to visualise the plaques. Isolated plaques were picked with a sterile Pasteur pipette into 1ml of TC-100 and stored at 4°C.

2.3.10.3 Amplification of virus stocks.

A 35mm tissue culture dish was seeded with 1×10^6 Sf9 cells in a final volume of 2ml. Following attachment of the cells to the plate, the medium was removed and replaced with 0.5ml of the viral inoculum prepared as described in section 2.3.10.2. The dish was incubated with gentle rocking for 1 hour at room temperature before adding 1.5ml of fresh medium to the dish and incubating at 27°C for 4 days. The medium containing numerous floating cells was centrifuged (1000g for 5 minutes) and the supernatant diluted with 3ml of medium stored at 4°C. This was designated the passage one viral stock.

Four 100mm tissue culture dishes were seeded with 5×10^6 Sf9 cells in a final volume of 10ml. The medium was removed and replaced with 1ml of the passage one stock and incubated with gentle rocking for 1 hour. 9ml of medium was added and the dishes transferred to 27°C for 4 days. The supernatant was collected as before and stored at 4°C in the dark. This was designated passage two stock.

2.3.10.4 Titration of virus stocks.

To determine the titre of the passage two virus obtained in section 2.3.10.3 virus was serially diluted and used to infect Sf9 cells according to the protocol described in section 2.3.10.2. Following staining of the plaquing overlay with Neutral Red, the plaques were counted and their numbers determined and expressed as plaque forming units per ml (pfu/ml).

2.4 RNA methods.

2.4.1 Isolation of total RNA from *P. falciparum*.

This method is based on the acidified guanidinium-phenol-chloroform protocol of Chomczynski and Sacchi (1987). *P. falciparum* cultures were grown to approximately 5×10^9 parasites, at which stage they were harvested by centrifugation (2500g, 10 minutes, 4°C). The red blood cell pellet was resuspended in ice cold 1 X SSC, 0.15% saponin and left to lyse on ice for 5 minutes. Parasites were washed in 1 X SSC and collected by centrifugation. The parasite pellet was resuspended in 7ml of solution D (4M guanidinium isothiocyanate; 25mM sodium citrate; 0.5% sodium lauryl sarosine; 100mM 2-mercaptoethanol), and repeatedly passed through a hypodermic needle to disrupt the pellet. 0.7ml of 2M sodium acetate pH 4.0, 7ml of water saturated phenol pH 5.0, and 1.4ml chloroform-isoamylalcohol (24:1 v/v) were added and mixed thoroughly. The solution was incubated on ice for 15 minutes before centrifugation (10,000g, 20 minutes, 4°C). The upper aqueous phase was quickly transferred to a clean tube and an equal volume of isopropanol was added before storing at -20°C for at least 2 hours to precipitate the RNA. The RNA was collected by centrifugation as above, and resuspended in 0.5ml of TE/0.1% SDS. The RNA was ethanol precipitated and resuspended in 0.2ml of TE/0.1% SDS, before storage at -70°C.

The RNA was analysed by UV spectrophotometry.

2.4.2 Isolation of yeast RNA.

This method is based on the protocol described by Higgins and Hames (1994) and involves the disruption of vegetative cells with hot phenol in the presence of SDS, which releases RNA effectively, but retains DNA within the cell wall.

200ml yeast cultures were grown to OD₆₀₀ 0.4-0.9 at which stage they were harvested by centrifugation for 5 minutes at 2000g. The cell pellet was resuspended in 2ml of sodium acetate-EDTA buffer (50mM sodium acetate, pH 5.3; 10mM EDTA), 0.25ml of 10% SDS w/v, and 2.5ml phenol equilibrated against sodium acetate-EDTA buffer. The solution was vortexed at maximum speed for 10 seconds then incubated at 65°C for 1

minute. The vortexing/heat treatment was repeated over a period of 5 minutes, then the solution was placed on ice for 10 minutes. The organic and aqueous phases were separated by centrifugation (2000g, 5 minutes) and the upper aqueous phase was quickly transferred to a clean tube. 2.5ml of phenol:chloroform (1:1 v/v) containing 0.5% 8-hydroxyquinoline and equilibrated against 10mM sodium acetate pH 6.0, 0.1M NaCl, and 1mM EDTA were added and the solution was mixed vigorously by hand shaking for 10 minutes. The phases were separated by centrifugation as before and the aqueous phase transferred to a clean tube. 2ml of chloroform:isoamylalcohol (24:1, v/v) were added and the phases mixed before centrifugation as before. The upper phase was transferred to a fresh tube and 0.1 volume of 3M sodium acetate pH 5.3 and 2.5 volumes of absolute ethanol were added to precipitate the RNA. The contents of the tube were mixed and stored for at least 1 hour at -20°C. The RNA was recovered by centrifugation (12,000g, 10 minutes at 4°C) and resuspended in 0.2ml of TE/0.1% SDS. The RNA was analysed by UV spectrophotometry. A further phenol chloroform extraction followed by ethanol precipitation was performed prior to reverse transcription reactions.

2.4.3 Isolation of insect cell RNA.

Two 100mm tissue culture dishes were seeded with 5×10^6 Sf9 insect cells. The cells were left to attach for 1 hour and the medium removed. Wild type AcNPV, or recombinant baculoviruses were added to the insect cells to give a multiplicity of infection of 20 (eg. 1×10^8 plaque forming units). The insect cells were incubated with virus for one hour with gentle rocking, before the viruses were removed by aspiration. Fresh medium was added and the dishes incubated at 27°C for 24 or 48 hours. Insect cells were harvested by scraping the cells off of the plates using a cell lifter. Cells were resuspended in fresh medium and pelleted by centrifugation (2000g, 5 minutes, 4°C).

The method used for isolation of RNA was the acidified guanidinium-phenol-chloroform protocol of Chomczynski and Sacchi (1987) described in section 2.4.1.

2.4.4 Reverse transcription.

Reverse transcription catalysed from an oligonucleotide annealed to a specific mRNA was used to generate a first-strand cDNA which could be used for a subsequent PCR reaction.

RNA samples were removed from -70°C and slowly defrosted on ice.

Storage buffer TE/0.1 % SDS was removed from the sample by ethanol precipitation. RNA samples were treated with DNase I prior to all reverse transcription experiments as such; the RNA pellet was resuspended in 10mM Tris-HCl, pH 7.5 with 10mM magnesium chloride. 1 unit of DNase I was added and the sample incubated for 10 minutes at 15°C. A sample of plasmid DNA was treated similarly to ensure that the DNase I reaction had worked.

The commercially available Pharmacia First-Strand cDNA synthesis Kit was used throughout. 5µg of total RNA was resuspended in 8µl of RNase free water, placed in a 1.5ml Eppendorf and heated to 65°C for 10 minutes before cooling on ice. 5µl of Bulk First-Strand reaction mix was added (45 mM Tris-HCl, pH 8.3; 68mM KCl; 15mM DTT; 9mM magnesium chloride; FPL *Cpure*^R Murine Reverse Transcriptase; RNAGuard; 0.08µg/ml RNase/DNase-Free BSA; 1.8 mM dATP; dCTP; dGTP and dTTP), along with 0.2µg of *NotI*-d(T)₁₈ primer.

The solution was mixed and incubated at 37°C for 1 hour at which stage the first-strand cDNA reaction was complete and could be used for subsequent PCR amplification.

2.4.5 Northern blots.

Total RNA was size fractionated on a 1% agarose/0.185% formaldehyde gel prepared and run in MOPS buffer (25mM MOPS; 5mM sodium acetate; 1mM EDTA, pH 7.0). Between 10-15µg of total RNA and 8µg of markers (0.24-9.4 kb RNA marker, GIBCO-BRL) were added to 0.15ml of loading buffer (2 X MOPS, 50% deionised formamide, 25% formaldehyde, 0.25% bromophenol blue and 0.25% xylene cyanol) and the sample was heated at 75°C for 10 minutes. The sample was snap cooled on ice for 2 minutes and 0.1µl of 1µg/ml ethidium bromide was added before loading to visualise the RNA with UV light. The samples were run at 100 V for around 3

hours with continuous buffer exchange by means of a peristaltic pump (Pharmacia LKB Pump P-1).

After photographing, the gel was washed once in 1 X SSC for 10 minutes to remove the formaldehyde, and washed again in 1 X SSC, 50mM sodium hydroxide to slightly denature the RNA.

The northern blot was set up as described in section 2.3.4.2 for Southern blotting. Following transfer of the RNA to the membrane, the membrane was prehybridised and hybridised to probe.

2.5 Protein methods.

2.5.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5.1.1 Preparation and running of SDS-gels.

Proteins were size fractionated under denaturing conditions using SDS-PAGE, with a discontinuous buffer system. Atto gel rigs (GRI Instruments) were used in conjunction with plates of 150mm X 150mm X 1mm size, and Bio-Rad Protean II rigs with plates of 250mm X 200mm. Mighty Small rigs (Hoefer) were used with plates of 80mm X 70mm X 0.75mm.

Table 3: protein gel mix for resolving SDS-PAGE

(quantities specified are in millilitres)

<u>Percentage Gel</u>	6%	7%	8%	10%
1.5M Tris-HCl, pH 8.8	15	15	15	15
Acrylamide/bisacrylamide 30:2.67	12	15	16	20
20% SDS	0.3	0.3	0.3	0.3
sdH ₂ O	32.7	29.7	28.7	24.7

The protein solution was degassed for 15 minutes to remove bubbles before adding 300µl of Temed and 30µl of 10% ammonium persulphate. The solution was cast to a level corresponding to approximately of 80% the length of the plate. 100µl of water saturated butanol was added to generate a level interface between the resolving and stacking gels. The gel was allowed to set for 1 hour prior to use.

Table 4: protein gel mix for stacking SDS-PAGE

(quantities specified in millilitres)

0.5M Tris-HCl, pH 6.8	5.0
Acrylamide/bisacrylamide 30:2.67	2.6
20% SDS	0.1
sdH ₂ O	12.3

The solution was degassed for 15 minutes to remove bubbles before adding 90 μ l of Temed and 18 μ l of 10% ammonium persulphate. The stacking gel was cast to the top of the plate and the comb was positioned before leaving the gel to polymerise for 1 hour before use.

Protein samples were added to an equal volume of 2 X SDS loading buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol and 0.25% bromophenol blue) and were boiled for 2-5 minutes before loading onto the gel. Gels were electrophoresed in running buffer (25mM Tris; 192mM glycine; 1% SDS) at 20 mA per gel and approximately 100 V, at constant current. Small gels were run for about 1 hour and large gels around 3 hours, until the blue tracking dye reached the bottom of the gel.

2.5.1.2 Protein size standards.

Three types of protein markers were used. For Coomassie blue stained gels, SDS-6H markers (Sigma), and for western blotting of gels pre-stained broad range markers (New England Biolabs) or pre-stained broad range Rainbow markers (Amersham).

2.5.1.3 Staining protein gels using fixing Coomassie blue stain.

Coomassie blue stain (0.25% w/v Coomassie brilliant blue R-250 prepared in water: methanol: glacial acetic acid, 5:1:1, v/v/v), was routinely used to visualise size fractionated proteins on SDS-polyacrylamide gels. Gels were incubated at 37°C in 200ml of Coomassie solution for 30 minutes before fixing/destaining the gel (water:methanol:glacial acetic acid, 5:1:1, v/v/v). The destain solution was replaced every 30 minutes until prominent protein bands became visible. The gels were dried under vacuum, against Whatman 3MM blotting paper at 80°C.

2.5.1.4 Staining protein gels using non-fixing Coomassie blue stain.

After electrophoresis, the gel was rinsed in several changes of distilled water, and incubated in 5 volumes of 0.05% Coomassie blue R-250 prepared in distilled water for 10 minutes. The gel was rinsed in several changes of distilled water until the proteins were visualised.

2.5.1.5 Isolation of proteins from SDS-polyacrylamide gels.

Proteins isolated this way were subsequently used for immunisation of rabbits to raise polyclonal antiserum.

Bacterial cells containing the recombinant protein were size fractionated by SDS-PAGE. The correct protein band was excised from the gel with the aid of non-fixing Coomassie blue stain to visualise the proteins (2.6.14). The protein gel slice was transferred to a clean tube and completely immersed in a solution of PBS/0.1% SDS and set at 4°C for 1-2 days. When all of the protein had leached from the gel, the solution was concentrated using a Microsept Filtron 10K microconcentrator which also removes SDS from the sample. The quantity and purity of the sample was assessed by SDS-PAGE.

2.5.2 Western blotting of SDS gels.

Proteins were separated by SDS-PAGE before western blotting by the method of Towbin *et al.* (1979) using HybondC (Amersham) nitrocellulose membranes. The SDS-polyacrylamide gel was removed from the glass plates and placed onto the nitrocellulose membrane and sandwiched between 2 sheets of blotting paper and 2 Scotchbrite pads each side. The gel sandwich was then submerged in transfer buffer (25mM Tris; 150mM glycine; 20% methanol and 0.1% SDS) in a transfer chamber TransBlot™Cell (BioRad) and blotted at 30mA/50V for 4 hours until the protein markers were completely transferred. The membrane was carefully incubated in blocking solution 1 X Tris/saline-5% fat-free milk powder w/v (2.2) for 1 hour. The primary antibody was added at the appropriate dilution in the same blocking solution and the membrane was rocked gently for 2 hours at room temperature. The membrane was given three washes to remove unbound antibody as such; 1 X Tris/saline

for 10 minutes (2.2); 1 X Tris/saline, 0.05% NP40; (2.2) twice for 10 minutes and 1 X Tris/saline for 10 minutes.

Goat anti-rabbit IgG-alkaline phosphatase conjugate or anti-mouse IgG-alkaline phosphatase conjugate (Promega) was added to the blocking solution at a dilution of 1:7500.

2.5.3 Isolation of cellular proteins.

2.5.3.1 Isolation of total protein from *P. falciparum* for analysis by SDS-PAGE.

Approximately 5×10^9 parasites harvested from synchronised or unsynchronised stage cultures were released from infected erythrocytes by saponin lysis (2.7.4) The parasites were collected by centrifugation (2000g, 10 minutes, 4°C) and washed in ice cold PBS to remove the red blood cell debris. The washed parasites were centrifuged (2500g, 5 minutes, 4°C) and lysed by resuspending the pellet in an equal volume of sdH₂O to which had been added 100µg/ml PMSF, 2µg/ml leupeptin, and 1µg/ml pepstatin A. An equal volume of 2 X SDS loading buffer was added to the sample. The lysate was passed repeatedly through a hypodermic needle to reduce the viscosity of the sample before boiling for 2 minutes prior to loading onto a large (Atto rig) SDS-polyacrylamide gel.

2.5.3.2 Isolation of total protein from *P. falciparum* for TopoII assays.

5×10^8 ring, trophozoite or schizont infected erythrocytes were saponin lysed on ice (2.7.4) then frozen at -70°C. Parasites were lysed on ice by the addition of 12µl of 50mM Tris-HCl pH 8, 120mM KCl, 10mM magnesium chloride, and 0.5mM each of ATP and DTT, to which 30µg/ml BSA, 1µg/ml pepstatin A, and 2µg/ml leupeptin had been added. The lysate was centrifuged (12000g, 10 minutes, 4°C) to remove insoluble cell debris.

2.5.4 Expression and isolation of recombinant proteins.

2.5.4.1 Expression and isolation of recombinant glutathione-S-transferase fusion proteins from *E. coli*.

Escherichia coli strain BL21 were transformed with recombinant PfTOP2 or parent pGEX2-T plasmids (2.3.6.2). A single colony harbouring either

the recombinant or parent plasmid was picked into 5ml of LB medium supplemented with 100µg/ml ampicillin (LB-Amp) and grown overnight at 37°C with shaking. The following day the culture was diluted 1:10 into fresh LB-Amp and grown at 37°C with shaking for 1 hour after which the culture was split into 1ml aliquots. The cells were induced with 0.1mM IPTG and grown for 1 to 4 hours. IPTG was omitted from one sample to allow comparisons to be made between uninduced and induced samples. 500µl of culture was collected using a benchtop microfuge and the supernatant discarded. 100µl of 1 X SDS loading buffer was added to the pellet before shearing the nucleic acids with a hypodermic needle to reduce the viscosity of the sample. The samples were then fractionated by SDS-PAGE.

2.5.4.2 Expression and isolation of recombinant hexahistidine fusion proteins from *E. coli*.

Escherichia coli strain BL21 (DE3) pLysS were transformed with PftOP2 recombinant plasmid and the parent plasmid (2.3.6.2). A single colony was picked into 5ml of minimal medium supplemented with 100µg/ml ampicillin-20µg/chloramphenicol (minimal-Amp-CMP) and grown overnight at 37°C with shaking. The culture was collected by centrifugation (2000g, 10 minutes) and the supernatant discarded. The pellet was washed in minimal medium and resuspended in fresh minimal-Amp-CMP in a fifth of the original volume. Half of the diluted culture was diluted 1: 50 in the same medium and grown at 37°C with shaking until an OD₆₀₀ of 0.7-0.8 was reached. An uninduced sample was removed and to the remaining culture 100mM IPTG was added to a final concentration of 0.5mM. The cells were incubated for an additional 1-4 hours before being collected by centrifugation using a benchtop microfuge. Pellets were either frozen at -20°C or analysed by SDS-PAGE.

2.5.4.3 Isolation of total protein from insect cells and baculovirus infected insect cells.

35mm tissue culture dishes were seeded with 1 X 10⁶ insect cells. The cells were allowed to attach to the dishes for 30 minutes before removing the

medium (TC-100 medium supplemented with 10% fetal bovine serum), and replacing it with recombinant, or wild type virus diluted to give 2×10^7 plaque forming units (pfu) in a total volume of 250-500 μ l. Viruses diluted in this way give a multiplicity of infection (MOI) of 20 pfu/cell. One plate was left without virus to use as a mock infected control. The viruses were incubated with the insect cells for 1 hour at room temperature with gentle rocking, before being aspirated from the dishes. Fresh medium was overlaid before incubating at 27°C for 18 to 72 hours. The medium containing 'floating' infected insect cells was removed from the dish and the cells were collected by centrifugation (2000g, five minutes, 4°C). The pellet was then resuspended in 50 μ l of 1 X SDS loading buffer. The dishes containing infected cells that were still attached to the dish were placed on ice and 50 μ l of 1 X SDS loading buffer was added and the lysate was scraped off using a cell lifter. Both lysates were used separately or combined together, and were stored at -20°C. The samples were then fractionated by SDS-PAGE.

2.5.4.4 Isolation of total protein from *S. cerevisiae* cells.

Six colonies were inoculated into yeast minimal medium containing 2% glucose/2%glycerol/2%lactate containing the appropriate amino acids (2.2) and grown for 24 hours at 30°C. Under these conditions the GAL1 promoter is repressed. 0.5 ml of this culture was added to two flasks each containing 9.5 ml of yeast minimal medium (2%glycerol/2%lactate and appropriate amino acid), and grown for an additional 24 hours at 30°C to deplete endogenous pools of glucose. When the OD₆₀₀ of the culture reached 0.4-0.9, one sample was induced with 2% galactose, and the other sample was allowed to continue to grow without galactose. This represented the non-induced control. Galactose induction was allowed to proceed for 2-6 hours.

Cells were harvested by centrifugation at 1500g for 5 minutes. The cell pellet was resuspended in 0.5 ml of water and centrifuged at top speed in a benchtop microfuge for 1 minute. An equal volume of acid-washed 400-600 micron glass beads was added to the pellet and cells were lysed by addition of 1 ml of Buffer 1 (2.2) per milligram of cells (wet weight) using

the following procedure. The mixture was vortexed for 20 seconds, placed in an ice water bath for 40 seconds. This was repeated ten times. Complete cell lysis was verified by microscopic analysis of the supernatant. The mixture was centrifuged for 1 minute at top speed in a benchtop microfuge, at 4°C and the supernatant removed. An equal volume of 2 X SDS loading buffer was added to each 100µl sample, before analysis by SDS-PAGE.

2.5.5 Metabolic labelling of proteins expressed in BL21 (DE3) pLysS.

Cells were grown as described in section 2.5.4.2 before removing 0.5ml aliquots into tubes 1-4. To tubes 3 and 4, 0.5mM IPTG was added and incubation at 37°C maintained for 30 minutes. 200mg/ml of rifampicin was added to tubes 2 and 4 and incubation continued for 45 minutes. Each of the samples were then pulse-labelled with 5µCi of ³⁵S methionine (1000Ci/mmol Tran³⁵S-label ICN) for 5 minutes at room temperature. The cells were collected using a benchtop microfuge and the pellets lysed by addition of 200µl of 1 X SDS-PAGE loading buffer. The samples were analysed by SDS-PAGE, the gels were fixed, soaked in ddH₂O for 15 minutes followed by soaking with 1M sodium salicylate for 15 minutes. The gels were then rinsed with water before vacuum drying and exposing to unflashed film in cassettes without intensifying screens until a clear signal was obtained.

2.5.6 Purification of hexahistidine-tagged proteins from baculovirus infected cells.

The commercially available HisTrapTMKit (Pharmacia) was used for purification of recombinant hexahistidine-tagged proteins.

2.5.6.1 Nickel column preparation.

A luer adaptor was connected to the syringe and filled with distilled water. The top cap from the column was unscrewed and a few drops of distilled water were applied to the top of the column to prevent air bubbles getting into the column. The twist-off end of the column was removed and 5ml of distilled water was forced through the column to

remove the stabilising agent. The column was activated with nickel ions by forcing 1ml of 0.1M nickel sulphate through the column. Excess nickel was removed by washing the column with 5ml of distilled water. The column was pre-equilibrated with 10ml of start buffer A (20mM disodium hydrogen phosphate-2H₂O; sodium dihydrogen-H₂O and 0.5M sodium chloride, pH 7.5; 20% glycerol and 10mM imidazole).

2.5.6.2 Sample preparation.

Between 250ml to 1.5 litre of recombinant infected baculovirus cells were harvested from spinner culture flasks. Cells were collected by centrifugation (500g, 5 minutes, 4°C) and the supernatant discarded. The pellet was resuspended in 5ml of ice-cold start buffer A to which protease inhibitors were added (2mg/ml leupeptin, 1mg/ml pepstatin and 100mg/ml PMSF).

Baculovirus cells were lysed by sonication using a 5mm diameter probe for 10-15 second bursts alternating with 30 seconds on ice. Cell lysis was checked by light microscopy. The viscosity of the sample was reduced by repeatedly passing through a hypodermic needle to shear the nucleic acids. The crude lysate was centrifuged (15000g, 30 minutes, 4°C) to remove insoluble material that could block the nickel column.

2.5.6.3 Affinity column purification.

All steps were carried out at 4°C. The nickel column was pre-equilibrated with 10ml of start buffer A before loading the sample by means of a peristaltic pump (Pharmacia LKB Pump P-1) at a flow rate of 1ml/minute. Fractions were collected in 0.5ml aliquots. The column flow through was retained and a small quantity combined with an equal volume of 1 X SDS sample buffer to be analysed by SDS-PAGE. The column was washed using 50ml of start buffer. Hexahistidine tagged proteins were eluted by the addition of 5ml of start buffer containing 0.5M imidazole, or eluted using a linear gradient of 10-500mM imidazole. Samples to be used for assays were stored on ice until required and were desalted by drop dialysis using a 0.1µM VC filter (Millipore) against TopoII assay buffer (2.9.1). Dialysed samples were analysed before and after dialysis using SDS-PAGE

to ensure that there was no loss of protein. Samples that were used for analysis by SDS-PAGE were added to an equal volume of SDS-loading buffer and stored at -20°C until required.

2.5.7 Indirect immunofluorescence assay (IFA).

Thin blood smears of infected erythrocytes from unsynchronised cultures at 6-10% parasitaemia were made on glass microscope slides. The slides were air dried for 1 hour before fixing with acetone for 5 minutes. After drying, a grid was drawn onto the slide with nail varnish. Onto each section of the grid 40µl of the appropriate antibody diluted in PBS was spotted and the slide and incubated in a moist chamber for 1 hour at room temperature. The slide was washed by immersion in PBS for 5 minutes twice, followed by one wash in distilled water. The slide was air dried and 40µl of a 1:80 dilution of a rhodamine isothiocyanate conjugate of immunosorbant-purified anti-rabbit immunoglobulin (RITC-anti-rabbit IgG, Sigma) containing 5µg/ml of DAPI in PBS was added to each grid section. The slide was incubated, washed and dried as described for the primary antibody. The slide was mounted in Citifluor (City University London) and examined by fluorescence microscopy using RITC filter set (Leitz).

2.5.8 Immunoprecipitation

2.5.8.1 Metabolic labelling of insect cells using ³⁵S methionine.

This method is based on O'Reilly *et al* (1992). 35mm culture dishes were seeded and infected with viruses as described in section 2.12. Two hours before harvesting the cells, 0.5ml of methionine-deficient TC100 medium supplemented with 10 fetal bovine serum (Gibco), was added to the cells to deplete the endogenous pools of methionine within the cells, after which they were transferred to 27°C for 1 hour. One hour before harvesting the cells, 0.5ml of the same medium containing 25µCi of 1000Ci/mmol Tran³⁵S-label (ICN) was added. After 1 hour, the radiolabelled culture medium was carefully removed and disposed of appropriately. The cells were washed gently three times in PBS pH 6.2, drained and 50µl of NP40 lysis buffer was added (1% NP40; 150mM NaCl;

50 mM Tris-HCl, pH 8.0) containing 2mg/ml leupeptin, 1mg/ml pepstatin and 100mg/ml PMSF). The lysate was scraped from each dish using a cell lifter.

2.5.8.2 Metabolic labelling of *P. falciparum* infected erythrocytes.

Prior to labelling the parasites, an enrichment procedure was used to facilitate selection of infected erythrocytes only to enhance metabolic labelling. The 3D7 isolate was used for this purpose because it exhibits a "surface knob phenotype" which alters the behaviour of the infected cells promoting their selection.

5 ml of infected erythrocytes at approximately 5-10% parasitaemia were collected by centrifugation (850g, at 20°C, for 10 minutes, with no brake). The medium was discarded, a blood smear taken and 2ml of complete RPMI medium was added. The volume of the cell pellet was determined and 2.4 volumes of plasmagel (Scottish Blood Transfusion Service), and 1.4 volume of RPMI were added (adjusting for the 2ml of RPMI already added prior to determination of the pellet volume). The sample was mixed and incubated at 37°C for 30 minutes. The supernatant containing the infected erythrocytes was transferred to a fresh tube and the number of infected cells determined using a counting chamber. The infected cells were collected by centrifugation as above, and a blood smear taken to determine the percentage of infected cells in the population. The cell pellet was washed twice in RPMI without methionine and cysteine (Sigma). 2×10^8 infected erythrocytes in a total of 2ml methionine- and cysteine-free RPMI were incubated with 100 μ Ci of 1000Ci/mmol Tran³⁵S-label (ICN), at 37°C for 1.5 hours, after being gassed. The cells were collected by centrifugation as above and the cell pellet washed with 10ml of PBS. The pellet was resuspended in 0.5ml of RPMI, the pellet volume determined, and the solution transferred to an eppendorff. At this stage the parasites were saponin lysed and the parasite pellet quick frozen and stored at -70°C.

2.5.8.3 Immunoprecipitation from insect cells.

Preparation of Protein A-Sepharose.

Protein A-Sepharose powder was swollen for one hour in swelling buffer (100mM Tris-HCl, pH 7.5), centrifuged (1000g, 2 minutes) before being washed twice in the same buffer.

Preclearing the sample.

2µl of pre-immune rabbit serum was added to 5µl of lysate and the sample incubated at 4°C for 1 hour. 10µl of Protein A-Sepharose CL-4B was added and the sample incubated at 4°C as before. The sample was spun in a microfuge for 2 minutes at 4°C and the pellet discarded. An additional 10µl of Protein A-Sepharose was added to the supernatant and incubated 4°C for 30 minutes. This procedure was repeated once more and the pellet discarded. The supernatant was considered now to be precleared.

Formation of the antigen-antibody complex.

2µl of the appropriate polyclonal antiserum was added to the precleared supernatant and incubated at 4°C for 3 hours.

Precipitation of antigen-antibody complexes.

50µl of pretreated Protein A-Sepharose was added to the complex and incubated at 4°C for 1 hour. The complexes were collected by centrifugation in a microfuge for 2 minutes and the supernatant discarded. The pellet was washed with 0.5ml of NET buffer (140mM NaCl; 50mM Tris-HCl, pH 8.0; 5mM EDTA; 0.05% v/v NP40) and vortexed briefly to resuspend the pellet before centrifugation as described above. This procedure was repeated twice. The washed antigen-antibody complexes were disrupted by resuspending the pellet in 1 X SDS loading buffer. Samples were size fractionated using SDS-PAGE. The radiolabelled gel was treated as described in section 2.5.5 prior to autoradiography.

2.5.8.4 Immunoprecipitation from *P. falciparum*.

Preparation of Protein A-sepharose.

Protein A-Sepharose beads were swollen (2.5.8.3.) then equilibrated with washing buffer A (10mM Tris-HCl, pH 7.5; 150mM NaCl; 1% v/v NP40 and 2mM EDTA) three times (1000g for 2 minutes).

Preclearing the sample.

A 1:1 ratio of sepharose beads to washing buffer was used throughout. One volume of solubilising buffer (50mM Tris-HCl, pH 7.5; 150mM NaCl;

1% NP40 and 4µg/ml PMSF) was added to 2×10^7 parasites, and the volume adjusted to 50µl. 2µl of pre-immune serum, and 35µl of sepharose beads (prepared as described above) were added and the slurry incubated at 4°C for 30 minutes. The slurry was spun in a benchtop microfuge at top speed for 1 minute and the supernatant collected. An additional 35µl of sepharose beads were added and the incubation and centrifugation steps repeated.

Formation of the antigen-antibody complex.

The supernatant was collected and 5µl of immune serum added and the solution incubated at 4°C for 1 hour on a rotating wheel. Following centrifugation in a benchtop microfuge, the supernatant containing the antigen-antibody complex was collected.

Precipitation of antigen-antibody complexes.

35µl of Protein A sepharose beads were added to the antigen-antibody complex and incubation continued for an additional 1 hour at 4°C on a rotating wheel. The Protein A sepharose complexes were collected by centrifugation in a microfuge as above prior to being washed 3 times in washing buffer A, twice in washing buffer B (10mM Tris-HCl, pH 7.5; 500mM NaCl; 1% NP40 v/v and 2mM EDTA) and once in washing buffer C (10mM Tris-HCl, pH 7.5). 25µl of 2 X SDS loading buffer was added and the samples analysed by SDS-PAGE, using a 10% polyacrylamide gel. Size fractionated proteins present in the gel were fixed using an aqueous solution containing 10% methanol v/v and 10% glacial acetic acid v/v, for 15 minutes. The gel was soaked in ddH₂O for 15 minutes, then soaked in a solution containing 1M sodium salicylate prepared in ddH₂O for 15 minutes. After rinsing in several changes of ddH₂O, the gel was dried under vacuum and exposed to film.

2.5.9 Complementation analysis in *S. cerevisiae*.

Temperature sensitive *top2-1* or *top2-4* yeast strains (2.1.6.2) were transformed as described in section 2.3.17.1 with recombinant or control plasmids. A single colony was inoculated into 100ml of yeast synthetic minimal medium with 2% glucose and appropriate amino acid supplements (2.2). Overnight cultures were grown at permissive

temperatures and cell numbers counted using a haemocytometer (Weber). Equivalent cell dilutions were spotted onto selective media (containing either, glucose, glycerol/lactate or galactose carbon sources at 2%) for each strain and the plates were transferred to permissive (25°C) or restrictive (34°C) temperatures.

2.6 Polyclonal antibody production in rabbits.

2.6.1. Raising polyclonal antisera against recombinant PftopoII hexahistidine- tagged protein.

2.6.1.1 Analysis of pre-immune sera.

New Zealand White rabbits were used for all antibody work conducted in this study. Animal work was conducted by the University of Edinburgh Medical Faculty Animal Area. To ensure that no cross-reactive antibodies were present in the rabbit serum prior to immunisation, a small (1.5ml) blood sample was taken from several animals for individual screening. Blood samples were placed at 37°C for 1 hour then transferred to 4°C overnight. Clear serum was removed from the blood clot and screened by IFA. Only animals with an acceptably low background of cross-reactive proteins were selected for subsequent immunisations.

2.6.1.2 Immunisation regime.

An additional 5ml of pre-immune blood was taken prior to the first immunisation. Protein for the first immunisation was prepared as described in section 2.11. Between 50-100µg of recombinant protein was added to 0.5ml PBS/0.5ml Freund's Complete Adjuvant. The emulsion was injected subcutaneously into each hind-quarter. Three weeks later the animal was boosted with 50-60µg of the same protein, but this time prepared in 0.5ml PBS/0.5ml Freund's Incomplete Adjuvant. Rabbits received three boosts in total. 10-14 days after each boost, a 9ml blood sample was taken from the ear. Blood samples were prepared as in section 2.6.1.1 and the immune response monitored by IFA and western blotting.

2.7 Parasite culture.

The KI isolate (Thiathong and Beale, 1981) and 3D7 isolate (Walliker *et al.*, 1987) of *P. falciparum* were used during the course of this work. Stocks were obtained from 1ml samples of ring stage infected erythrocytes stored in liquid nitrogen.

2.7.1 Human blood and serum.

Blood and serum used for this work were obtained from the Southeast Scotland Blood Transfusion Service. Whole blood packs containing group O rhesus-positive blood were aliquoted into 50ml tubes (Falcon) and washed 3 times in 5 volumes of sterile RPMI 1640 (Gibco), with centrifugation between washes (1500g, 10 minutes, 4°C). The white blood cell layer on top of the erythrocytes was carefully removed following each spin. The erythrocytes were resuspended in RPMI 1640 to give a 66% haematocrit, and were stable up to 4 weeks stored at 4°C. Human serum packs were pooled and centrifuged (2000g, 10 minutes, 4°C). The serum was collected and heat treated at 56°C for 1 hour. 50ml aliquots were stored at -20°C and were stable for at least 1 year.

2.7.2 Culturing of the blood stages of *P. falciparum*.

The method used in this study was originally described by Trager and Jensen in 1976 and was later modified by Zolg *et al.*, 1982. Parasites were cultured in 75cm³ tissue culture flasks (Falcon 3024) in 50ml of complete medium (RPMI 1640 supplemented with 10% human serum, 50µg/ml gentamycin and hypoxanthine). The percentage parasitaemia was obtained by taking thin blood smears and fixing briefly in microscopy grade methanol (BDS) prior to staining with a 10% solution of Giemsa (BDH) prepared in distilled water. Cultures were maintained at 1-2% except when building up for RNA or protein preparations when up to 10% parasitaemia was acceptable. The medium was changed on a daily basis by aspiration, the percentage parasitaemia determined, fresh blood and medium added and the parasites gassed (3% oxygen; 2% carbon dioxide and 95% nitrogen) and incubated at 37°C. 4ml of infected blood per flask was not exceeded.

2.7.3 Synchronisation of blood stage parasites.

Parasite cultures containing at least 5% ring stage parasites were synchronised by sorbitol treatment as described by Lambros and Vandenberg (1979).

Medium was aspirated from the parasites and infected blood collected by centrifugation (850g, 10 minutes, 4°C). The blood pellet was washed once in fresh RPMI 1640 and centrifuged as before. The infected blood was incubated for 5 minutes at room temperature in a solution of 5% sorbitol and the blood collected as before. The pellet was washed twice in 5 volumes of RPMI 1640 and resuspended in complete medium at 5% haematocrit. The synchronised culture was gassed and returned to a 37°C incubator (2.7.2). The culture was examined 48 hours later to determine the degree of synchrony and if necessary, the process was repeated again to achieve tighter synchronisation.

2.7.4 Saponin lysis of blood stage parasites.

P. falciparum cultures were harvested by centrifugation (2500g, 10 minutes, 4°C). The red blood cell pellet was resuspended in ice cold 1 X SSC, 0.15% saponin and incubated on ice for 5 minutes. Red blood cell lysis was confirmed by detection of a colour change from red to a dark reddish black. Parasites were washed in 1 X SSC or PBS and collected by centrifugation. Pellets were used immediately or stored frozen at -70°C until required.

2.8 Culture of *Spodoptera frugiperda* (Sf9 cells).

S. frugiperda insect cells were cultured in 50ml tissue culture flasks (Falcon) which permit attachment of cells to the plate. Routinely, insect cells were sub-cultured 1:2-1:8 when they reached 80-90% confluency, in 50ml fresh TC100 medium supplemented with 10% FBS and 50µg/ml gentamycin. Insect cells were also grown in suspension culture in 1 litre glass bottles containing a hanging bar in-built magnetic stirrer (Techne) at 60rpm (MCS-104S Biological Stirrer -Techne). Insect cells were maintained at 27°C.

2.9 Enzyme assays.

2.9.1 Decatenation assays (Parasite extract).

Parasite extract was prepared from synchronised cultures containing ring, trophozoite and schizont parasites as described in section 2.7.3. 5×10^8 parasites were harvested by saponin lysis as described in section 2.7.4. Parasite pellets were stored frozen and lysed on ice by addition of ice-cold TopoII assay buffer supplemented with $1\mu\text{g/ml}$ pepstatin A, $100\mu\text{g/ml}$ PMSF and $2\mu\text{g/ml}$ leupeptin. Insoluble cell debris was removed by centrifugation ($12,000g$ 4°C , 5 minutes).

Typically, $0.2\mu\text{g}$ of kinetoplast DNA (KDNA) was used per $20\mu\text{l}$ assay. Reactions containing TopoII assay buffer (50mM Tris-HCl, pH 8.0; 120mM KCl; 10mM magnesium chloride; 0.5mM each of DTT, ATP, and $30\mu\text{g/ml}$ of BSA) were initiated by variable amounts of enzyme or parasite extract (typically 1×10^6 parasites gave good results) and were incubated for 30 minutes at 37°C . Reactions were terminated with 0.1 volume of $2 \times$ loading buffer, loaded onto 1% agarose gels containing $0.5\mu\text{g/ml}$ ethidium bromide and electrophoresed for variable periods of time. Reaction products were visualised by UV irradiation. One unit of topoisomerase II is defined as the amount required to completely decatenate $0.2\mu\text{g}$ of KDNA in 15 minutes at 37°C .

2.9.2 Immunodepletion of PfTopoII activity.

Parasite extract was prepared from unsynchronised cultures as described in section 2.7.4. Parasite pellets were lysed on ice as described in section 2.9.1. Pre-immune or immune sera were incubated with parasite extract prepared from 1×10^8 parasites at dilutions in the range of 1:5- 1:2000 for 30 minutes on ice. Decatenation assays were conducted and the reaction products analysed as detailed in section 2.9.1.

2.9.3 Decatenation assay (recombinant PfTopoII).

Fractions obtained from imidazole gradient purifications of recombinant PfTopoII were dialysed against TopoII assay buffer to remove imidazole and salt (2.5.6.3). $0.2\mu\text{g}$ of kinetoplast DNA (KDNA) was used per $20\mu\text{l}$ assay. Reactions containing TopoII assay buffer (50mM Tris-HCl, pH 8.0;

120mM KCl; 10mM magnesium chloride; 0.5mM each of DTT, ATP, and 30µg/ml of BSA) were initiated by variable amounts of partially purified protein and incubated for 30 minutes at 37°C. Reactions were terminated using 0.1 volumes of 2 X loading buffer, loaded onto 1% agarose gels containing 0.5µg/ml ethidium bromide and electrophoresed for variable periods of time.

Chapter 3

Heterologous expression of *P. falciparum* topoisomerase II in *E. coli*.

3.1 Introduction.

There were two main objectives of the work described in this chapter.

(1) *To express a fragment of the PfTOP2 gene in E. coli to use to make polyclonal antiserum in rabbits.*

Figure 1 shows a schematic alignment of the predicted domain structure of type II topoisomerases from both prokaryotic and eukaryotic species. In *S. cerevisiae*, V8 proteinase sensitive sites occur after glutamate residues 410, and 680, the latter of which is conditional upon ATP binding. In addition, the region around residue 1200 is also sensitive to V8 digestion; this maps the beginning of the C-terminal domain, which is reported to be species divergent. A comparison of the predicted amino acid sequence of *S. cerevisiae* and *P. falciparum* topoisomerases II reveals an identity of 44.4% between the two homologues. The degree of conservation is generally greater in the amino terminal two-thirds of the proteins and falls off markedly towards the carboxy termini. For this reason, it was decided to express the last 404 amino acids of PfTopoII for use as an antigen, in the expectation that antibodies raised against this region would be less likely to cross-react with topoisomerase II present in the cells of any organism that was subsequently used for heterologous expression of PfTopoII. The expression vector to be used was pRSETB.

(2) *To express the entire PfTOP2 gene in E. coli.*

The full length PfTOP2 gene was simultaneously prepared for expression in pRSETB. A different expression vector, pGEX-2T was also used for full length expression of the gene. Both vectors are commonly used for heterologous expression of foreign proteins in *E.coli*.

Figure 1.

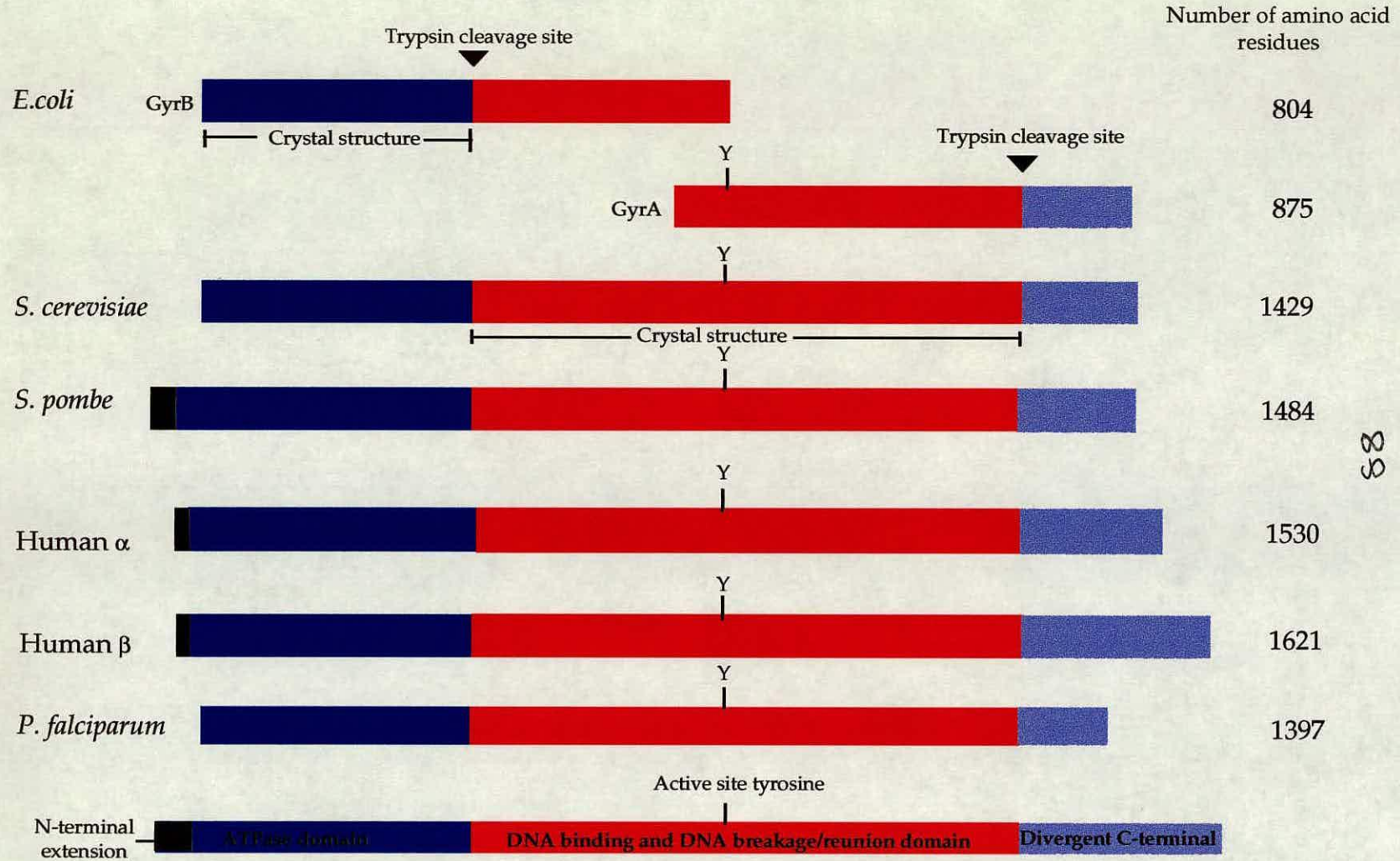
Domain structure of topoisomerase II homologues from different species.

A diagrammatic comparison of the domain structures of *E. coli* DNA gyrase and five eukaryotic topoisomerases II. Gyrase is composed of two polypeptides, A and B, which in the functional enzyme are in the A₂B₂ configuration. All eukaryotic homologues are composed of single polypeptides which associate to form a homodimeric enzyme.

The ATP binding domain is shown in dark blue, the central DNA binding and strand breakage/reunion domain is shown in red, the species divergent carboxyterminal domain is shown in pale blue, and N-terminal extensions are shown in black. Each protein is aligned by the active site tyrosine (residue 829 in the *P. falciparum* homologue). Domain boundaries have been denoted by reference to protease-sensitive sites within the *E. coli* or *S. cerevisiae* proteins.

Figure 1 is adapted from a review article by Watt and Hickson (1994).

Figure 1. Domain structure of topoisomerase II homologues from different species.



Background to the pRSET system.

The pRSET vector system (Figure 2) uses a strong phage T7 promoter to drive expression of the target fragment cloned in-frame downstream of the vector encoded ATG. Six histidines immediately downstream of the ATG encode an N-terminal metal binding domain which can be cleaved from the recombinant protein by means of an adjacent enterokinase site. Stop codons are present in all three frames within 70 nucleotides of the polylinker. The nickel binding domain can be used to affinity purify the recombinant protein using nickel column chromatography.

Background to the pGEX system.

pGEX vectors express a fragment of foreign DNA as a fusion partner to glutathione S-transferase (GST) of *Schistosoma japonicum* (Figure 3). The *lac* repressor binds to the P_{tac} promoter, repressing expression of the fusion protein, which can be reversed by the inducer IPTG. The gene of interest is cloned in frame into the polylinker downstream of the end of the GST gene. Termination codons are present in all three frames at the end of the polylinker. pGEX-2T contains a thrombin cleavage site to enable the GST moiety to be cleaved from the fusion protein.

3.2 Expression of the C-terminal region of PfTopoII in pRSETB.

A 3' 1.2 kb (2979-4191 nt) fragment of the PfTOP2 gene, previously cloned into pUBS1 was released by restriction with *Bam*HI and *Bgl*II. The fragment was gel purified and ligated in frame into the *Bam*HI site of pRSETB. *Bgl*II and *Bam*HI have compatible sticky ends making it possible to clone the fragment into the *Bam*HI site.

The ligation was transformed into competent DH5 α cells and recombinant colonies were detected by hybridisation to the ³²P random labelled PfTOP2 fragment (2979-4191 nt) described above. Because cloning into the *Bam*HI site of pRSETB could result in ligation of the fragment in two different possible orientations, the cloning boundaries were checked by sequencing to ensure that the fragment was in-frame with the vector-

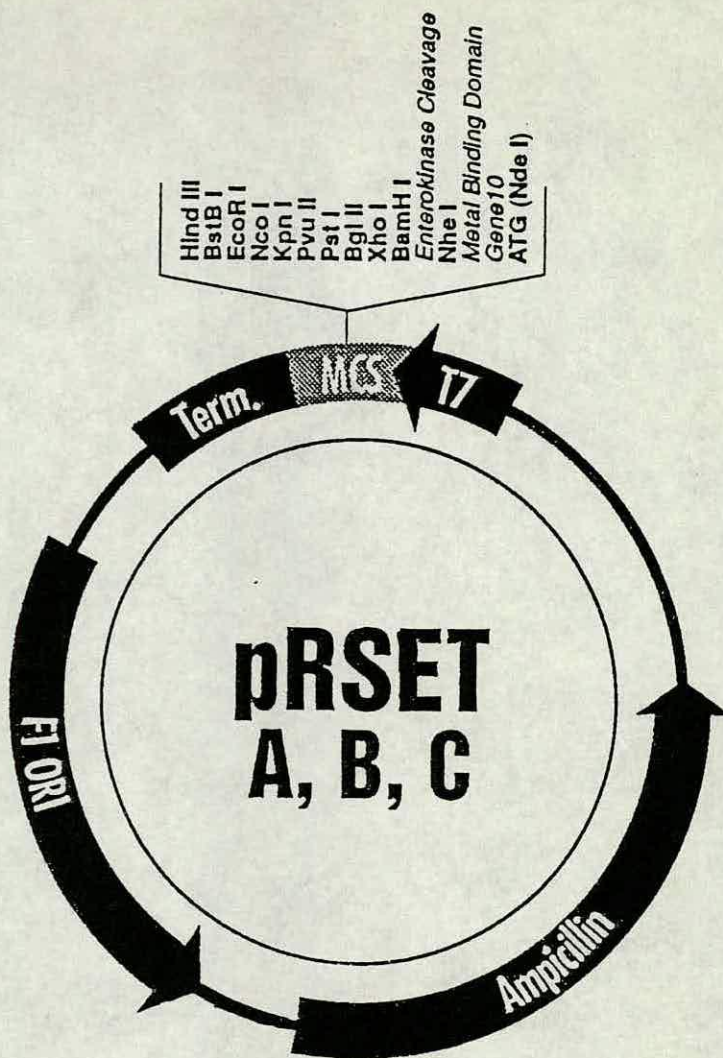


Figure 2. Map of the pRSET series of expression vectors.

The elements for the control of transcription consist of a ribosome binding site downstream of a strong phage T7 promoter. The vector encoded ATG is followed by a few vector encoded amino acids plus a hexahistidine nickel binding domain. Stop codons in all three frames are present within 70 nucleotides of the multiple cloning site (MCS) which is followed by a transcription terminator.

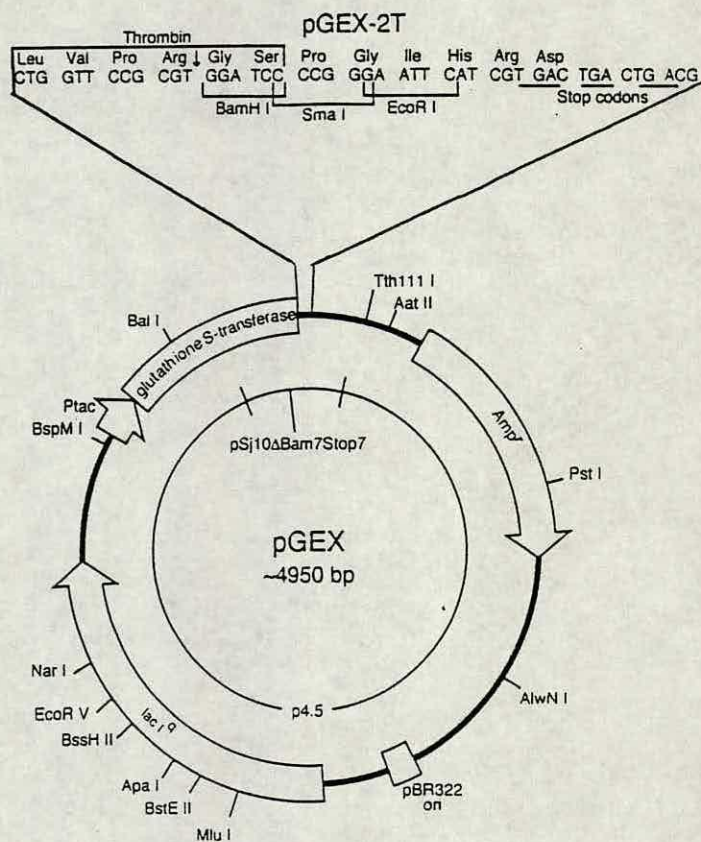


Figure 3. Map of the pGEX-2T expression vector.

The plasmid contains a mutant *LacI* gene (*LacI^q*) which produces high levels of the lac repressor which binds to the P_{tac} promoter repressing expression of the GST gene. The foreign gene is inserted into the multiple cloning site at the 3' end of the GST gene. Stop codons are present in all three frames downstream of the distal *EcoRI* cloning site. Expression of the GST fusion protein is induced with IPTG. A thrombin cleavage site facilitates removal of the GST moiety following expression.

encoded ATG. Suitable recombinants were transformed into BL21 (DE3) [pLysS] for expression.

BL21 cells were chosen because they lack the *lon* protease and the *ompT* outer membrane protease that can degrade proteins during purification. DE3 is a λ phage derivative that carries the gene for T7 RNA polymerase (gene 1) under the control of the *lacUV5* promoter (Studier and Moffat 1986). Addition of IPTG induces the *lacUV5* promoter to produce T7 RNA polymerase, which in turn transcribes any DNA under the control of a T7 promoter. Transcription by the basal level of T7 RNA polymerase present in the non-induced cell can be reduced by the presence of plasmid pLysS (Studier, 1991). This plasmid provides the cell with T7 lysozyme which is a natural inhibitor of T7 RNA polymerase.

3.2.1 Evidence of expression of the 54.5 kDa recombinant C-terminal region of PfTopoII.

In order to test for expression of the fragment, individual transformants harbouring the recombinant plasmid, or the parent plasmid alone, were inoculated into minimal medium and grown to an OD₆₀₀ of 0.7-0.8. Cultures were grown in the presence or absence of IPTG for periods of 1 to 4 hours (2.5.4.2). Small samples were removed and tested by SDS-PAGE fractionation for the presence of a novel band of around 54.5 kDa, the size predicted from the composition of the amino acids encoded by the DNA fragment. A faint band in the size range expected was observed in recombinant preparations only (Figure 4).

3.2.2 Confirmation of expression of the C-terminal region of PfTopoII.

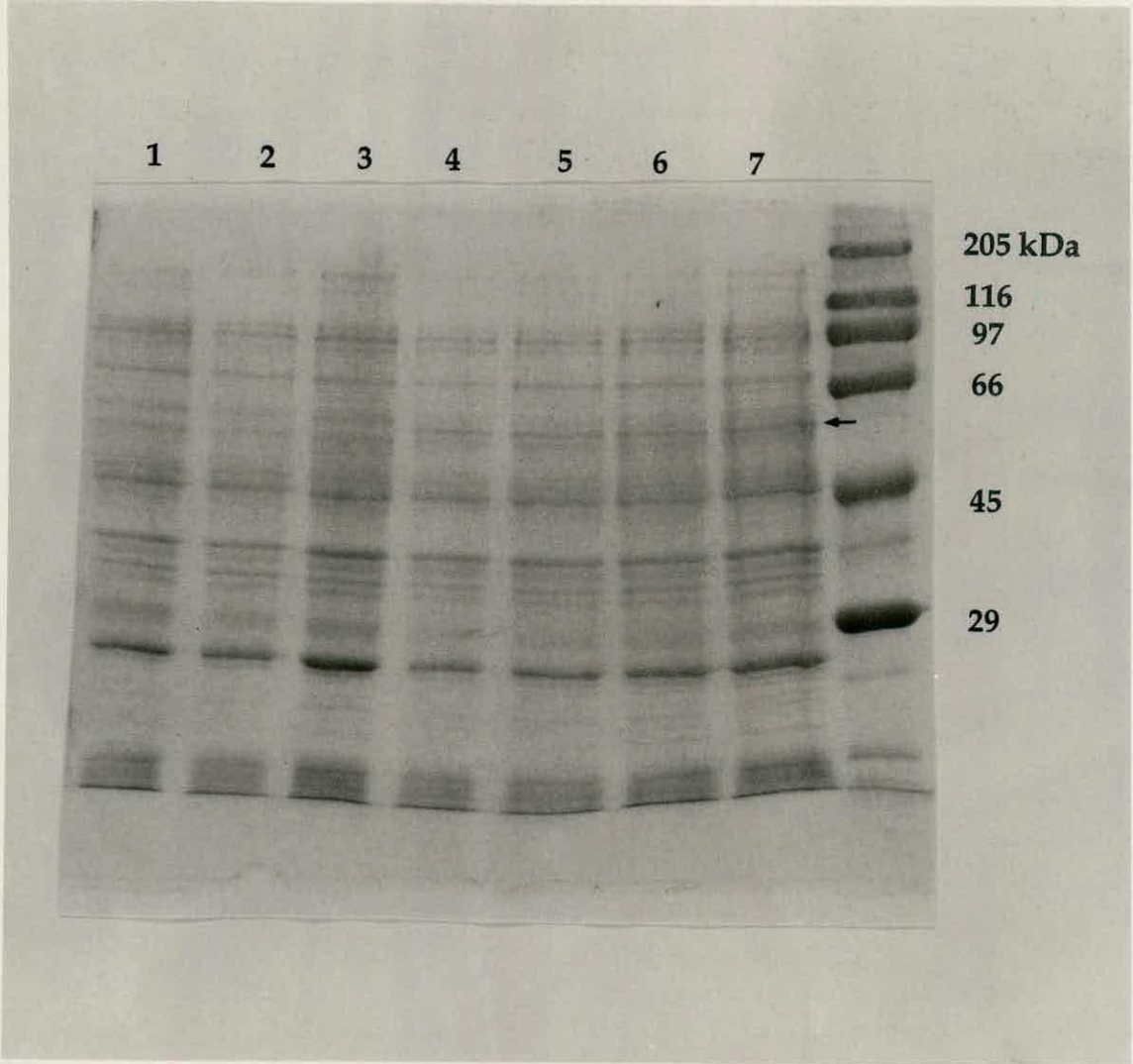
To verify that the protein was derived from expression from the T7 promoter, a pulse labelling experiment was conducted. This involved adding ³⁵S methionine to cells grown in minimal medium without amino acids for 5 minute periods. This enables efficient labelling of all newly synthesised proteins containing methionine residues. If the experiment is performed in the presence of rifampicin (which inhibits *E. coli* RNA polymerase), then only proteins that are transcribed from the T7 promoter by T7 RNA polymerase will be labelled.

Figure 4.

Coomassie blue stained SDS-polyacrylamide gel of size fractionated proteins from *E. coli* cells harboring the 3' 1.2 kb fragment of the PftOP2 gene (2979-4191 nt) in pRSETB (pRSETB/3' PftOP2).

Lanes 1 and 2 contain proteins derived from expression of the parent plasmid pRSETB without the PftOP2 gene fragment. Lane 3 contains pRSETB/3' PftOP2 in non-induced cells. Lanes 4-7 contain pRSETB/3' PftOP2 in cells induced with IPTG for 1-4 hours, respectively.

The position of the protein size standards are indicated in kilodaltons and the position of the recombinant protein is arrowed.



Small scale cultures containing the recombinant plasmid having reached an OD₆₀₀ of 0.8, were pulse labelled using ³⁵S methionine in the presence or absence of IPTG or rifampicin, or in the presence of both (2.5.5.).

Figure 5 (a) shows the results of this experiment. In the absence of both rifampicin and IPTG, radiolabelled host cell proteins can be clearly seen (lane 1). In the presence of rifampicin only, the appearance of a faint band of the expected size is observed, which may be attributed to leaky expression (lane 2). Following addition of both IPTG and rifampicin to the culture, however, a major protein of approximately 50-60 kDa together with several other smaller proteins are observed (lane 4). These small polypeptides could represent degradation products and/or truncated versions of the major protein.

3.2.3 Purification of the C-terminal region of PfTopoII.

In order to obtain enough pure protein to immunise rabbits, the recombinant protein was isolated directly from SDS-polyacrylamide gels. Total cell protein from a large scale induced culture, was size fractionated using SDS-PAGE on two 7% gels. The gels were stained with non-fixing Coomassie blue in aqueous solution and the appropriate band was excised. Protein was eluted from the gel in PBS/0.1% SDS. The recombinant protein was concentrated and the SDS removed with a microconcentrator, and the purity of the sample confirmed by SDS-PAGE. Two New Zealand White rabbits designated 989 and 992 were each immunised with 100µg of recombinant purified protein emulsified in 0.5ml Freund's complete adjuvant and 0.5ml of PBS. Pre-immune serum was taken prior to the first immunisation. The immunisation regime described in section 2.6.1.2 was followed and immunisations were performed by qualified individuals under Home Office Regulations.

3.3 Primary screening of antisera

Serum obtained following second and subsequent immunisations were tested for the presence of PfTopoII specific antibodies. This was done by screening western blots of the antigenic hexahistidine fusion protein immobilised on nitrocellulose with appropriate dilutions of the immune

Figure 5 (a). Autoradiograph of metabolically labelled cells.

Equal numbers of *E. coli* BL21 (DE3) [pLysS] cells harboring the 3' 1.2 kb *Bam*HI/*Bgl*III fragment of the PfTOP2 gene in pRSETB were treated with IPTG, rifampicin, or a combination of both prior to analysis by SDS-PAGE (10% gel).

Lane 1. ³⁵S methionine labelled culture. Lane 2. ³⁵S methionine labelled culture previously treated with rifampicin. Lane 3. ³⁵S methionine labelled culture previously treated with IPTG. Lane 4. ³⁵S methionine labelled culture previously treated with rifampicin + IPTG.

The positions of the protein size standards are indicated and the position of the 50-60 kDa protein of interest is arrowed.

Figure 5 (b) Western blot of antigenic fusion protein.

Western blot analysis of whole cell lysate following size fractionation by SDS-PAGE (10% gel). Lanes 1 and 2 contain BL21 (DE3) [pLysS] cells expressing recombinant C-terminal PfTopoII that was used as the antigen. The blot was probed with immune serum obtained following the second immunisation. Cross reactive proteins were detected using anti-rabbit IgG alkaline phosphatase conjugate.

Lane 1. Strip from western blot probed with serum from rabbit 989 at a dilution of 1:1000.

Lane 2. Strip from the same western blot probed with serum from rabbit 992 at a dilution of 1:1000.

Protein markers are shown to the left of lane 1.

(a)

Rifampicin	-	+	-	+
IPTG	-	-	+	+
	1	2	3	4

200 kDa

97

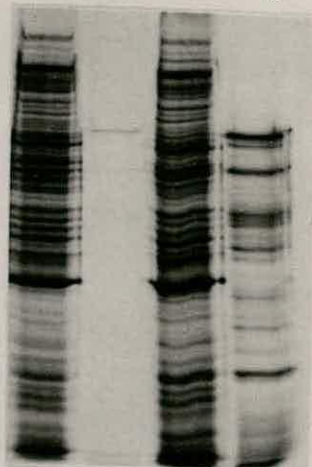
69

46

30

21

14



(b)

200 kDa

97

69

46

30

21/14



97

69

serum.

Figure 5 (b) shows the result of one of these experiments. Lane 1 and 2 are strips taken from the same western blot and probed with immune serum from rabbit 989 (lane 1) and rabbit 992 (lane 2). Each rabbit serum appears to detect a similar panel of proteins, the largest of which is estimated to be approximately 55-60 kDa. A comparison with Figure 5 (a) shows that the proteins recognised by each rabbit on the western blot are very similar to the pattern of expression from the T7 promoter (Figure 5 a). Taken together, these results indicate that both rabbits have produced polyclonal antiserum against the recombinant C-terminus of PfTopoII.

3.3.1 Western blot analysis of parasite extract identifies a protein of around 160/175kDa.

A protein with an apparent mobility of around 160/175 kD was detected by each antiserum at a dilution of 1:1000 on western blots of size fractionated parasite proteins (10% SDS-PAGE) obtained from unsynchronised blood-stage cultures (Figure 6). The pre-immune serum failed to recognise parasite protein(s) on western blots.

3.3.2 PfTopoII localises to the parasite nucleus.

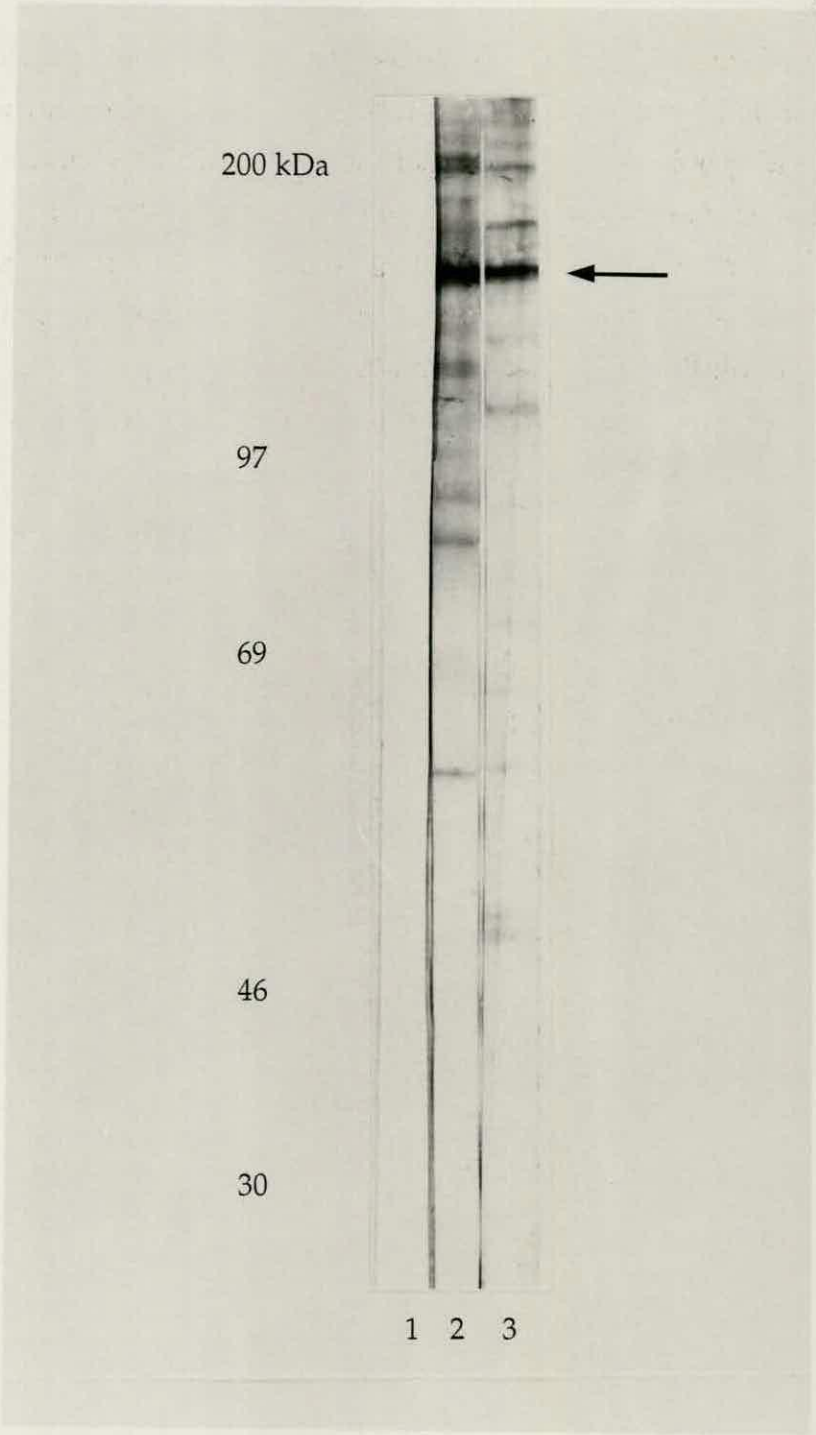
Figure 7 (1) shows a thin smear of parasitised blood displaying the intraerythrocytic stages of *P. falciparum* (ring, trophozoite and schizont) taken from a culture containing human red blood cells. The parasites were stained with Giemsa (2.7.2).

Antisera (from rabbits 989 and 992) were used at a dilution of 1:80 in PBS to screen thin blood smears of parasitised human erythrocytes in an immunofluorescence assay (IFA, 2.5.7) that were fixed to glass slides (Figure 7, panels 2 and 3). Pre-immune serum, diluted similarly, was also tested and PfPCNA antiserum (Horrocks *et al.*, 1996) was used as a comparison as it is known to localise to the parasite nucleus and therefore acted as a positive control (shown in Chapter 4, page 130 b). IFAs showed that the pre-immune serum from each rabbit failed to cross-react with intraerythrocytic parasites, in contrast with the signals from each of the C-terminal PfTopoII antisera which co-localise with the nuclear stain DAPI

Figure 6.

Western blot of size fractionated proteins prepared from unsynchronised parasites separated by SDS-PAGE on a 10% gel. Lane 1. Strip from western blot probed with pooled pre-immune serum from rabbits 989 and 992 at a dilution of 1:1000. Lane 2. Strip from from the same western blot probed with immune serum from rabbit 989 at a dilution of 1:1000. Lane 3. Adjacent strip from western blot probed with immune serum from rabbit 992 at the same dilution as in lanes 1 and 2.

The positions of the protein size standards are indicated in kilodaltons and the position of PfTopoII is arrowed.



100

64

Figure 7.

1.

Thin blood smear of methanol-fixed and Giemsa-stained *P. falciparum* parasites in human red blood cells. A. Ring stage parasite. B. Trophozoite stage parasite. C. Schizont stage parasite.

2 and 3.

Indirect immunofluorescence assay of a trophozoite stage parasite.

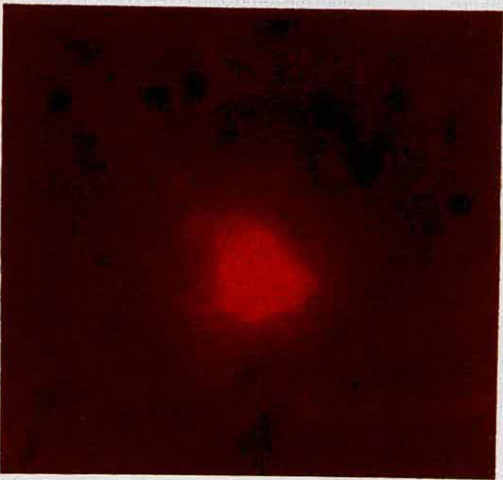
Acetone fixed thin blood smears from unsynchronised parasite populations were probed with polyclonal antiserum from rabbit 989 (panel 3). Cross-reactive parasites were detected using anti-rabbit IgG RITC conjugated second antibody. Parasite nuclei were counter-stained with DAPI (panel 2) and rhodamine (panel 3).



1



2



3

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in trophozoites and schizont stages. Ring stage parasites gave a weaker level of fluorescence (result shown in Chapter 4, page 130 b).

3.3.3 A triplet of proteins of around 160/175 kDa can be immunoprecipitated from parasite extracts.

Immunoprecipitation was used to determine whether PfTopoII interacts with other parasite protein(s). A set of pilot experiments were performed using the parasite isolate 3D7. This isolate exhibits a "knob phenotype" which affects the behaviour of infected red blood cells when immersed in a solution of plasmagel.

Infected erythrocytes were separated from uninfected cells using plasmagel which causes uninfected erythrocytes and ring stage infected erythrocytes to settle at the bottom of the plasmagel solution, leaving trophozoite and schizont infected erythrocytes in suspension. 80% enrichment of the culture for these stages was achieved, enabling metabolic labelling of parasite proteins to be performed in a small volume thus promoting efficient incorporation of label.

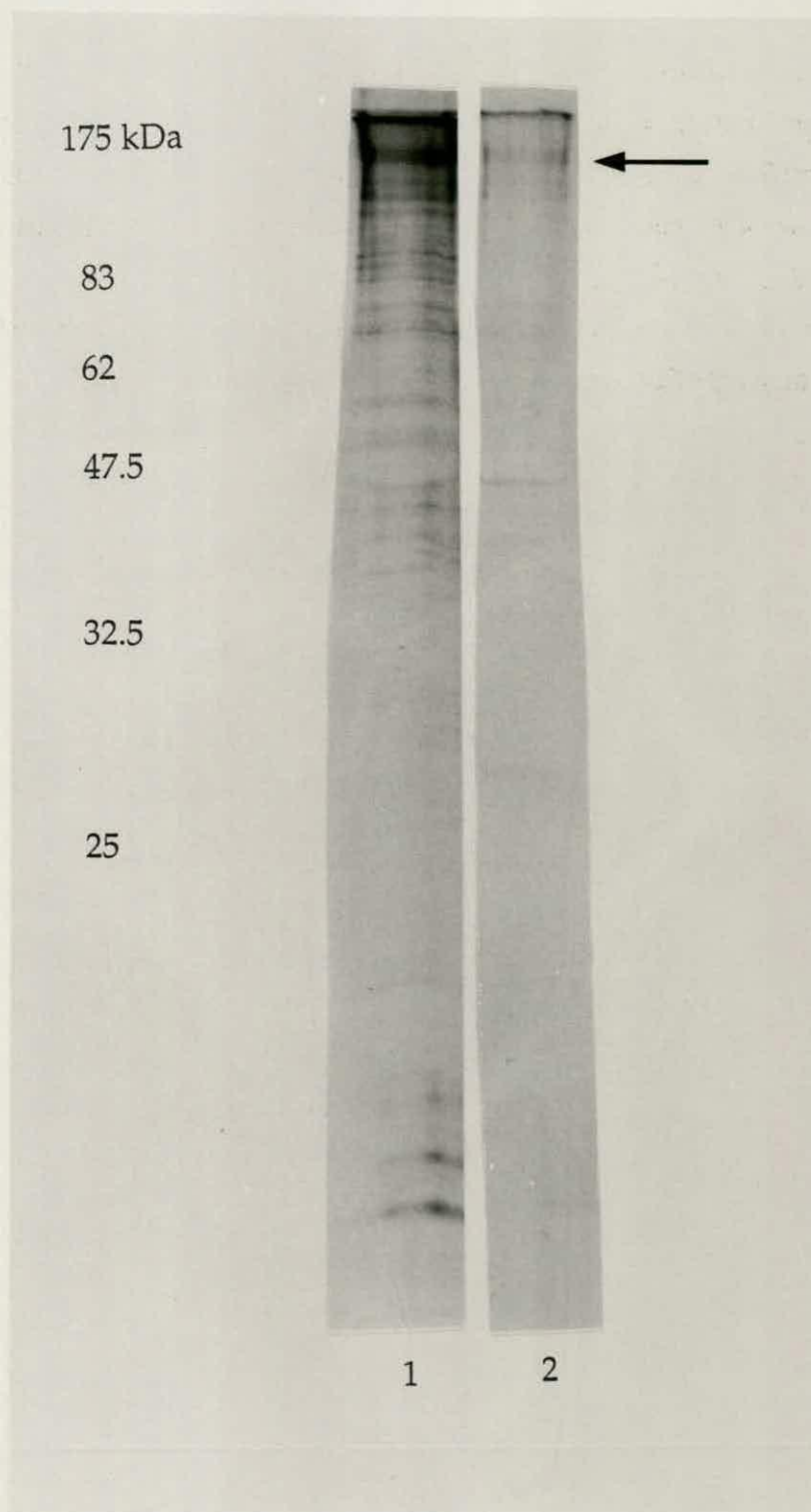
Enriched cultures were incubated in methionine/cysteine deficient RPMI medium in the presence of ^{35}S methionine/cysteine for 1.5 hours, after which the parasites were harvested by saponin lysis (2.5.8.2). PfTopoII was immunoprecipitated using the PfTopoII antiserum from rabbits 989 and 992. This technique exploits the specific binding of antibody with its cognate antigen; antigen binds to the antibody via two antigen binding sites present on the two Fab regions of the antibody. Addition of Protein A sepharose beads, which bind to the Fc portion of antibody, enable the complex to be precipitated. SDS-PAGE fractionation, of the precipitated complex in the presence of β -mercaptoethanol disrupts the protein complexes and separates them according to their size and subunit composition.

Figure 8 shows that a triplet of bands (the broad band arrowed in figure 8) is present in the immunoprecipitated complex with an apparent mobility of ~160/175 kDa. This size is in agreement with previous results obtained from western blots of size fractionated parasite proteins (figure 6). Several other bands are also present in addition to the triplet. Their identities

Figure 8. Immunoprecipitation of PfTopoII from crude parasite extract.

10% SDS-polyacrylamide gel fractionated immunoprecipitated complexes. Unsynchronised blood stage parasites were metabolically labelled using ^{35}S methionine prior to immunoprecipitation using immune serum from rabbits 989 and 992.

Lane 1. Immunoprecipitation using immune serum from rabbit 989. Lane 2. Immunoprecipitation using immune serum from rabbit 992. The positions of the protein size standards are indicated in kilodaltons. The position of the protein triplet is arrowed and appears as a broad band in this photograph.



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have not yet been investigated further, but they may represent proteolytic degradation products of the full length protein.

3.4 Expression of the entire PfTOP2 gene in pRSETB.

The *Bgl*II DNA fragment comprising the complete coding sequence of the PfTOP2 gene (1-4191 nt) that was originally prepared for expression in baculovirus (described in Chapter 5) was subcloned in frame into the *Bam*HI site of pRSETB.

The ligation was transformed into *E. coli* DH5 α competent cells and recombinant colonies were detected by hybridisation to the ³²P random labelled PfTOP2 fragment (2979-4191 nt) described above. Because cloning into the *Bam*HI site of pRSETB could result in ligation of the fragment in both possible orientations, the cloning boundaries were sequenced to ensure that the fragment was correctly orientated in-frame with the vector encoded ATG. Suitable recombinants were transformed into BL21 (DE3) [pLysS] for expression.

3.4.1 Initial characterisation of protein expression.

BL21 (DE3) [pLysS] harbouring the PfTOP2 gene in pRSETB were grown to an OD₆₀₀ of 0.8, prior to induction with IPTG for periods of 1-4 hours. Cells were harvested by centrifugation, lysed, and analysed by SDS-PAGE to monitor expression. Uninduced cultures containing the PfTOP2 gene, together with cultures containing the parent plasmid alone were used as controls.

The PfTOP2 gene encodes a protein of 160 kDa/subunit. No evidence of a product of this size was observed after Coomassie blue staining of SDS protein gels (data not shown). At this time the commercially available monoclonal antibody against the hexahistidine tag of fusion proteins was not available, nor was antiserum against the recombinant C-terminus of PfTopoII. In an effort to establish whether the full length protein was being expressed, therefore, a metabolic labelling experiment was performed.

Small scale cultures containing the recombinant plasmid having reached an OD₆₀₀ of 0.8 were pulse labelled using ³⁵S methionine in the presence

of IPTG or rifampicin alone, or in the presence or absence of both.

Figure 9 (a) shows the results of this experiment. Lanes 1-4 are derived from cultures containing the parent pRSETB plasmid without the PfTOP2 gene. Lane 5-8 are derived from cultures containing the PfTOP2 gene in pRSETB. In the presence of rifampicin and IPTG there is little evidence of expression of a protein of the expected size of recombinant PfTopoII (Lane 8). There are some very faint bands that are not observed in the culture derived from the parent plasmid (without the PfTOP2 gene) that was also treated with rifampicin and IPTG (lane 4). The presence of these proteins of approximately 90, 50 25 and 20 kDa may indicate that (i) the protein is undergoing premature transcriptional and/or translational termination *in vivo* or (ii) the full length protein is made in small amounts but is rapidly degraded *in vivo*.

3.5 Expression of the PfTOP2 gene in pGEX-2T.

In view of the fact that full length expression of the PfTOP2 gene was not observed in pRSETB, it was decided to see if changing the expression system could achieve better results. pGEX was considered worth trying for two reasons. Firstly, this system expresses foreign proteins as C-terminal fusions to GST. Because GST is expressed at a high level and appears to be very stable, it was hoped that this would improve the stability of recombinant PfTopoII expressed as a fusion protein. Secondly, a rabbit polyclonal antiserum against GST was commercially available which would simplify detection of GST fusion proteins. GST fusion proteins can also be readily purified using glutathione Sepharose columns.

pGEX-2T does not contain a *Bgl*III site so the *Bgl*III fragment of the PfTOP2 gene (1-4191 nt) was cloned in frame into the *Bam*HI site; this was possible because both enzymes generate compatible sticky ends. Ligations were transformed into competent DH5 α cells and colonies screened for the presence of the PfTOP2 gene by colony hybridisation. Cloning boundaries were sequenced to ensure that the gene was in the correct orientation and in frame with the vector-encoded ATG.

Small cultures grown at 37°C containing the recombinant PfTOP2 gene in pGEX-2T or the parent vector alone were induced with IPTG (2.5.4.1).

Figure 9 Autoradiograph of metabolically labelled cells.

(a)

Equal numbers of *E. coli* BL21 (DE3) [pLysS] cells metabolically labelled with ^{35}S methionine and harboring the PftOP2 gene in pRSETB were treated with IPTG, rifampicin, or a combination of both prior to analysis by SDS-PAGE (10% gel).

Lanes 1 and 5. ^{35}S methionine labelled culture with no supplements. Lanes 2 and 6. ^{35}S methionine labelled culture previously treated with rifampicin. Lanes 3 and 7. ^{35}S methionine labelled culture previously treated with IPTG. Lanes 4 and 8. ^{35}S methionine labelled culture previously treated with rifampicin + IPTG.

The positions of the protein size standards are indicated. The positions of the faint bands which may represent proteolytic fragments of recombinant PftopoII are arrowed.

Figure 9. Western blot.

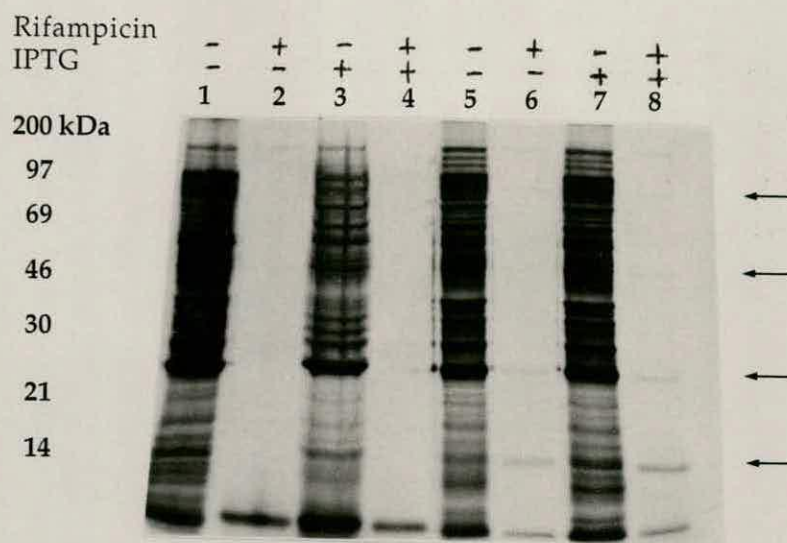
(b)

Western blot of size fractionated proteins (8% gel) from *E. coli* BL21 cells harboring the wild type PftOP2 gene and a mutated version of the gene in pGEX2T. In the mutated gene tyrosine 829 has been changed to phenylalanine by PCR mutagenesis. The blot was probed with the commercially available polyclonal antiserum against GST at a dilution of 1:1000.

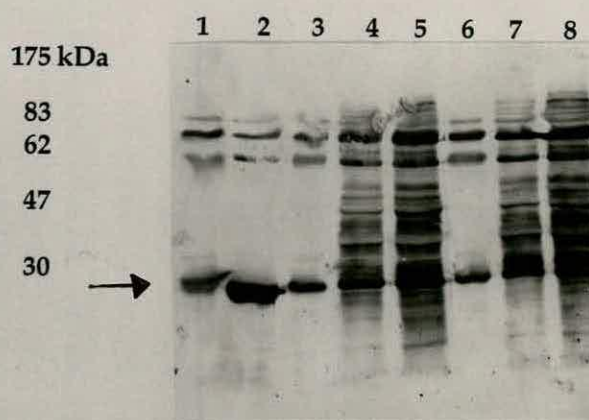
Lanes 1 and 2. Proteins from BL21 cells containing parental pGEX2-T vector alone. Lanes 3-5. Proteins from BL21 cells containing the wild type PftOP2 gene in pGEX2T. Lanes 6-8. Proteins from BL21 cells containing the mutated version of the PftOP2 gene. Lanes 1, 3 and 6 are non-induced cultures. Samples run in lanes 2, 4 and 7 were induced for 2 hours, whilst samples run in lanes 5 and 8 were induced for 4 hours.

The positions of the protein size standards are indicated, and the position of GST is arrowed.

(a)



(b)



Samples were taken prior to induction to act as controls. Cultures were grown for 1-4 hours in the presence or absence of IPTG before being analysed by SDS-PAGE (8% gel) using Coomassie blue to stain the gels or by western blotting.

Figure 9 (b) shows a western blot analysis of this experiment. Immobilised proteins were probed with anti-GST polyclonal serum at a dilution of 1:1000; cross reactive proteins were detected using anti-rabbit IgG alkaline phosphatase conjugate at 1:7500. Lanes 1, and 2 contain protein derived from the culture containing pGEX-2T without the PfTOP2 gene. Lanes 3, 4 and 5 contain protein derived from a culture containing pGEX-2T with the PfTOP2 gene. In lanes 1-3 the presence of a predominant protein of around 27 kDa is detected by the anti-GST serum. This protein is most likely to be GST on the basis of the expected size of 27.5 kDa and reactivity with anti-GST serum. Addition of IPTG to the culture containing the PfTOP2 gene construct results in the appearance of numerous GST-cross reacting proteins of 100 kDa or less. These are present after 2 hours induction and are still present 2 hours later (lanes 4 and 5 respectively).

Several possible explanations may account for this result. The fact that so many discrete protein bands appear to be cross reacting with the anti-GST serum suggests that full length PfTopoII is probably made, but that is undergoing C-terminal degradation. Alternatively truncated versions of the protein may have been expressed. It is possible that expression of the recombinant protein may be deleterious or toxic to the cell, a situation which might lead to *in vivo* proteolytic degradation.

3.5.1 Mutational replacement of the PfTOP2 active site tyrosine 829 with phenylalanine.

The possibility that the activity of this protein is toxic to *E. coli* in its own right, was investigated by introducing a mutation in the triplet encoding the tyrosine residue implicated in the DNA/protein linkages. The TAT triplet encoding tyrosine in the wild type enzyme was converted to TTT (phenylalanine) using overlapping primer extension (figures 10 and 11) PCR using primers G3718/T1728 amplified a product of approximately 1 kb, which is close to the size expected.

Figure 10.

Structure of the oligonucleotides that were used to change the active site tyrosine codon to phenyalanine in the wild type PftOP2 gene by PCR mutagenesis.

(i) sense top strand
oligonucleotide
T1729

5' GAT GCA TCT GCA GCA AGA **TTT** ATA TTT ACA AAA TTG GCT AGC 3'
D A S A A R **F** I F T K L A S

(ii) antisense
bottom strand
oligonucleotide
T1728

5' GCT AGC CAA TTT TGT AAA TAT **AAA** TCT TGC TGC AGA TGC ATC 3'

(iii) wild type
coding sequence

5' GAT GCA TCT GCA GCA AGA **TAT** ATA TTT ACA AAA TTG GCT AGC 3'
D A S A A R **Y** I F T K L A S

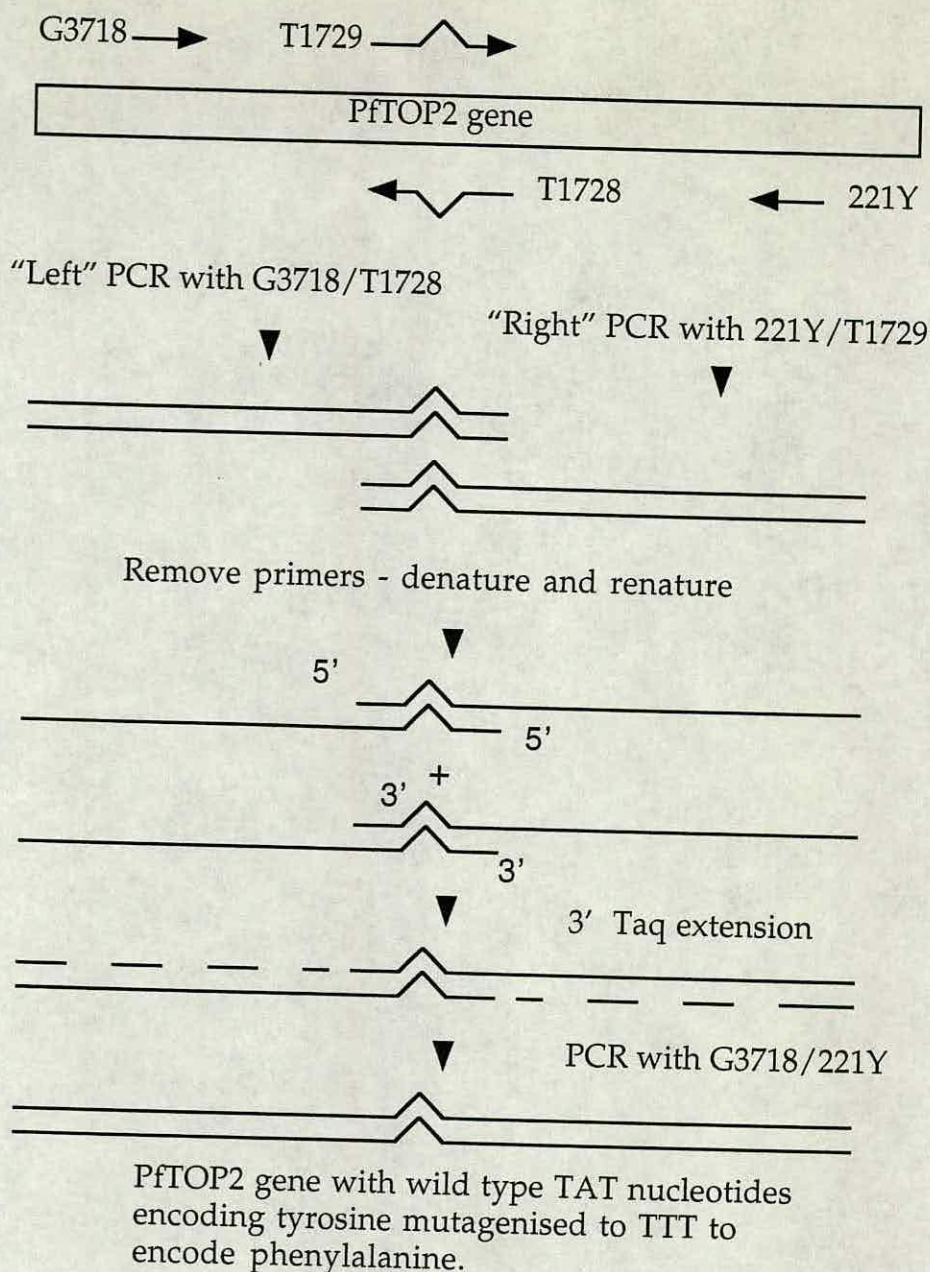


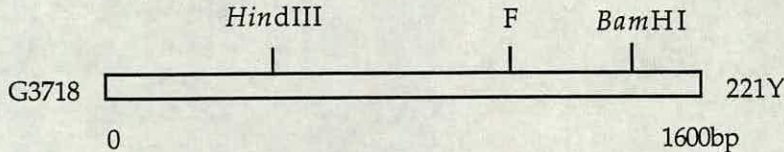
Figure 11.

Strategy used to replace the PfTopoII tyrosine 829 with phenylalanine by PCR mutagenesis.

This involved combining two separate PCR products with overlapping sequence into one longer product. Two overlapping primers T1728 and T1729 are shown containing three mismatched bases to the PfTOP2 gene.

PCR using primers 221Y/T1729 amplified a product also of the expected size (600 bp).

The two PCR products were gel purified, denatured at 95°C for 5 minutes, cooled to 37°C for 3 minutes, prior to 3' extension using Taq and dNTPs at 72°C for 10 minutes. A 1.6 kb 3' amplification product was subcloned into the pCRTMII vector and the ligation transformed into INV α F' competent cells (2.3.15.4).



Diagrammatic representation of the PCR fragment cloned into the pCRTMII vector.

Recombinant clones were sequenced to verify that the tyrosine codon had changed to phenylalanine and to ensure that no other mutations were present in the sequence. Part of the sequence containing the TAT to TTT (shown underlined) codon change obtained from the ABI 377 automatic sequencer (Perkin Elmer) is shown below.

		110	120	130	140	150
1	26•Sandie	GATGCATCTG	CAGCAAGATT	<u>TATATTTACA</u>	AAATTGGCTA	GCTCAACCAG
		160	170	180	190	200
1	26•Sandie	AAGTATATTT	AATGAATACG	ACGATCCTAT	TTTAAATAT	TTAAATGAAG

The *Hind*III/*Bam*HI fragment containing the mutation was restricted, gel purified and used to replace the wild type *Hind*III/*Bam*HI fragment of the gene. The mutated PfTOP2 gene was cloned into pGEX-2T in frame as described previously in section 3.8. Small scale cultures were analysed for expression of the PfTOP2 gene as also described previously.

Figure 9 (b) shows the results of this experiment. Lanes 6-8 are derived from a culture containing the mutated PfTOP2 construct in pGEX-2T. Lane 6 shows that in the absence of IPTG there is little evidence of

expression of the gene. IPTG induction of an aliquot of cells taken from the same culture however, results in a similar profile as that observed in cultures expressing the unmutated PfTOP2 gene (figure 9 b, lanes 7 and 8). This indicates that expression of a functional topoisomerase II is not in itself responsible for the result observed when attempts were made to express the wild type gene. However, it is possible that expression of the PfTopoII itself is detrimental to cell growth and viability.

3.6 Expression of the N-terminal region of the PfTOP2 gene in pRSETB

Figure 12 shows the amino acid structure of PfTopoII predicted from the coding sequence of the gene. The position of the C-terminal fragment (404 amino acids) that was expressed in the vector pRSETB is indicated. As part of a separate study, we were able to overexpress an N-terminal fragment of PfTopoII which comprised amino acids 6-144. This construct terminates before the polyasparagine tracts (not shown in figure 12). In view of the fact that both the N-terminal and C-terminal regions of the protein can be expressed, but the full length wild type and mutant forms of the protein appear to have a very short half-life *in vivo*, lead us to question whether the presence of the polyasparagine tracts might be detrimental to expression in *E. coli*. In addition, we were also interested to know which fragments of the protein could be expressed in *E. coli*. To investigate this we decided to express the *Bgl*III to *Hind*III restriction fragment (nucleotides 1-1797) of the PfTOP2 gene which encodes amino acids 1-599 of the protein.

The expression vector pRSETB was restricted with *Bgl*III and *Hind*III and the cut vector gel purified to increase the efficiency of ligation. The vector and gene fragments were ligated, and transformed into *E. coli* strain DH5 α . Suitable recombinant plasmids were isolated and transformed into *E. coli* strain BL21 (DE3) [pLysS] for expression.

3.6.1 Characterisation of expression.

BL21 (DE3) [pLysS] harbouring the 5' end of the PfTOP2 gene (nucleotides 1-1797) in pRSETB were grown to an OD₆₀₀ of 0.8, prior to induction with IPTG for periods of 1-4 hours. Cells were harvested by centrifugation,

lysed, and analysed by SDS-PAGE to monitor expression. Proteins were visualised by staining with Coomassie blue. Uninduced cultures containing the 5' end of the PfTOP2 gene, together with cultures containing the parent plasmid alone were used as controls.

Figure 13 shows the result of a typical experiment. The recombinant hexahistidine fusion protein was expressed at levels detectable by Coomassie blue staining. Its apparent mobility was estimated to be 68-72 kDa, a size which is consistent with the amino acid composition of the protein. Although expression of the recombinant protein was observed after 1 hours induction, expression was maximal after 4 hours induction. This result demonstrates that the presence of polyasparagine tracts in the PfTopoII protein does not appear to affect protein expression.

1 MAKNTIEER YQKKSQIEHI LLRPDTYIGS VEMHTQLLWV WNKEKNRMVQ
51 KNITYVPGLY KIFDEIIVNA ADVKAREKEK SENPMTCIKI EINKENKRIS
101 VYNDGEGIPV DIHKEMNIYV PHMIFGELLT SDNYDDAEDR ITGGRNGFGA
151 KLTNIFSKEF IVQCGDSSRK KEFKMTWSDN MSKFSEPHIK NYNGKDYVKV
201 TFKPDLNKFG MTEMDDDIES LLFKRVYDLA GTCsvrvylN GQRLAVKDFK
251 SYVDLYLKDN SNDNKNNKGQ NDNNNNNNNN NDENANQNND NLDVSLSNEP
301 ADGTPTKNNN NNNNNNDEDE IVKIHEKQHR WEIVVSKSDG SQFQQVSFVN
351 SICTTKGGSH VNYIVEQLLS SLSKKANAKN KGGMEIKSGH IRNHLWVFN
401 CLIVNPTFDS QTKETLTTPK VKFGSKCILS DKTINNVLKS PILSNILLWA
451 QAKAQVELKK KMKAGSSKAR ERIIGIPKLE DANDAGSKYS QECTLILTEG
501 DSAKTSLAG LSIVGRDKYG VFPLKGKLLN VRDASFQQLM DNKEIQNIFR
551 IMGLDITDKN KDDIKGLRYG SLMIMTDQDY DGSNIKGLLI NMIHKFWPSL
601 LKHKGFLSEF VTPIVKVQKG SQEYSFFTIA EYEQWKENTN LLGWKIKYYK
651 GLGTSTDREF KQYFSDIKNH KIMFLWTGDR DGDSIDMAFS KKRIEDRKLW
701 LQNFILGSYV DHKEKDLSYY DFNKELIYY SRYDTERSIP NIMDGWKPQG
751 RKVLYGCFKR NLRNECKVAQ LVGYIAEHSa YHHGESSLQQ TIINMAQTFV
801 GSNININFLEP CGQFGSRKEG GKDASAARYI FTKLASSTRS IFNEYDDPIL
851 KYLNEEGQKI EPQYYIPVIP TILVNGCEGI GTGYSSFIPN YNYKDIIDNI
901 KRYINKEPLI PMVPWYKDFK GRIESNGKTG YETIGIINKI DNDTLEITEL
951 PIKWTQDYK EFLEELLTDE KHQLILDYID NSSHEDICFT IKMDPAKLQK
1001 AEEGLEKVF KLKSTLTTTN MTLFDPNLKL QRYSTELDIL KEFCYQRLKA


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1051  YENRKSYLIS KLEKEKRIIS NKTGFILAIV NNELIVNKKK KKVLEELYR
1101  KGYDPYKDIN KIKKEEIFEQ ELLDAADNPE DNEEIIAGIT VKDYDYLLSM
1151  PIFSLTLEKV EDLLTQLKEK ERELEILRNI TVETMWLKDI EKVEEAIEFQ
1201  RNVELSNREE SNKFKVARKQ GPSSMKKKKK KKKLSSDEES EGGDTSDSSE
1251  FLVNTLNIKK NTNKKTTTSS NNVNNSKKRL RKADDLNSNE LDNTLSVSKT
1301  FDDNNNLTDN TPLINRLNDE NNEFSSNNVD NKSTNKNSRK KKPFIADSTN
1351  DNNSELNSSI QINDNVNDDI NITISPNTI NVNEFSSIKN KLELGI*

```

Figure 12. The amino acid structure of PfTopoII predicted from the coding sequence of the gene.

The N-terminal fragment that was expressed in pRSETB (pRSETB/5' PfTOP2) as part of this study, is shown in red. The polyasparagine rich regions which have been demonstrated to be present in the mRNA are indicated in blue.

The C-terminal fragment which was expressed in pRSETB (pRSETB/3' PFTOP2) is shown in purple.

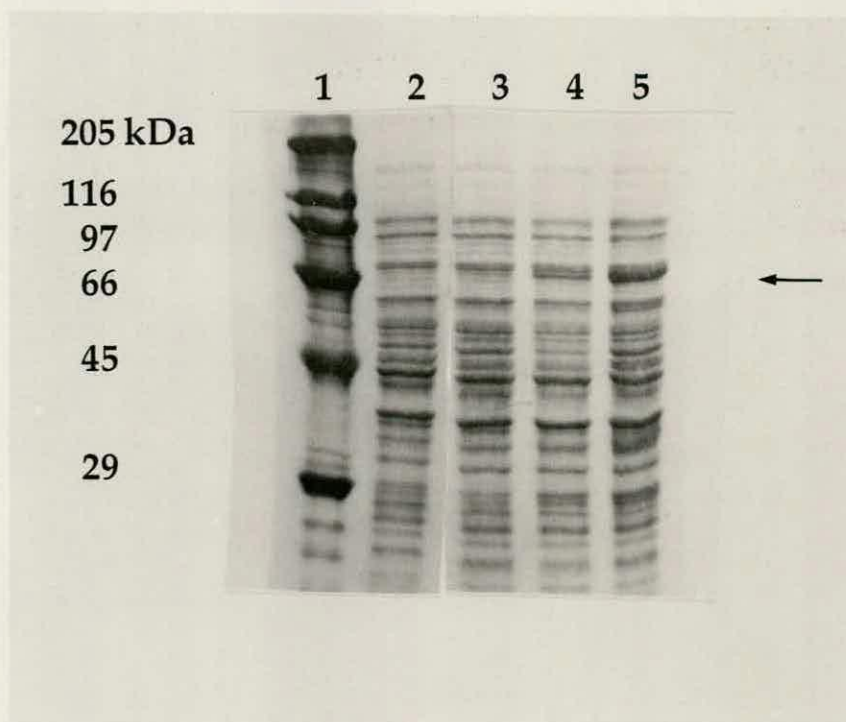
The glycine residue (147) implicated in ATP binding is shown in green, as is the active site tyrosine residue (829).

Figure 13.

Coomassie blue stained SDS-polyacrylamide gel of size fractionated proteins from *E. coli* cells harboring the 5' fragment of the PfTOP2 gene (nucleotides 1-1797) in pRSETB (pRSETB/5' PfTOP2).

The position of the protein size standards, indicated in kilodaltons are shown in lane 1. Lane 2 contains proteins derived from expression of the parent vector pRSETB without the PfTOP2 gene fragment. Lane 3 contains the pRSETB/5' PfTOP2 construct in non-induced cells. Lanes 4 and 5 contain the pRSETB/5' PfTOP2 construct in cells that had been induced for 1 and 4 hours respectively.

The position of the recombinant protein is arrowed.



3.7 Discussion

Antiserum raised against the C-terminal domain of PfTopoII in two rabbits (989 and 992) both recognise a protein of approximately 160 kDa on western blots of total parasite protein. Both antisera were also able to immunoprecipitate a triplet of proteins of similar size from parasite extracts. Strong patterns of immunofluorescence with trophozoites and schizonts were also obtained with each antiserum, but weaker fluorescence was evident with ring stage parasites. The region of fluorescence produced by the antibodies co-localised with the nuclear stain DAPI, corroborating earlier work which suggested that PfTopoII is a nuclear protein (Cheesman *et al.*, 1994). Both antisera also cross-react with the antigenic fusion protein that was used to immunise the rabbits.

Although both the N-terminal and C-terminal fragments of PfTopoII could be made in *E. coli*, full length expression of the gene was unsuccessful. No evidence of full length recombinant protein was observed when the gene was co-expressed with T7 RNA polymerase in PRSETB from a T7 promoter. When induced cultures were metabolically labelled in the absence of *E. coli* RNA polymerase activity (rifampicin inhibited), some very faint proteins were detected (approximate sizes 90, 50, 25 and 20 kDa), but their nature, whether degradation products, or truncated versions of the full length protein has not been determined.

Full length expression of the gene was also attempted using the pGEX-2T plasmid. The advantage of using this system is that PfTopoII could be expressed as a fusion partner with GST which is reported to increase the stability of some foreign proteins. When protein expression was monitored using a commercially available antiserum against GST, numerous cross-reactive bands of approximately 100 kDa or less were detected following induction. The possibility that expression of a functional topoisomerase II may have had a deleterious effect on cell viability, which in turn may have given rise to proteolytic degradation of the recombinant protein was investigated. This involved the use of PCR mutagenesis to change the active site tyrosine to phenylalanine, so that an inactive TopoII could be expressed. When this construct was expressed, however, a similar pattern of protein expression was observed.

The fact that other *P.falciparum* genes (eg. DNA polymerase δ and PCNA-*pers comm.*) have been successfully expressed in *E. coli* suggests that the high A+T content of *falciparum* genes in general does not present a barrier to expression. It seems more likely that recombinant PfTopoII is expressed in *E. coli*, but that it is rapidly degraded *in vivo*. Time limitations have precluded investigating these findings in greater depth.

Chapter 4

Expression of PfTopoII throughout the blood stages of parasite growth.

Introduction 4.1.

Several of the genes encoding parasite proteins involved in DNA replication have been isolated and characterised in this laboratory in an endeavour to provide new potential drug targets for development.

These include PfPCNA, PfPol δ , PfPol α and topoisomerase I (Kilbey *et al.*, 1993, Ridley *et al.*, 1991, White *et al.*, 1993, Tosh and Kilbey 1995), the first three of which are homologues of proteins which in the SV40 system are known to act directly at the replication fork. Polymerase δ is responsible for achieving continuous leading strand synthesis in conjunction with its processivity factor PCNA. Synthesis of Okazaki fragments is initiated by the polymerase α /primase complex, but polymerase δ /PCNA complete the elongation phase after complex switching (Stillman, 1994).

As part of this work, the stage specific expression of three of these genes has been examined and some interesting observations have been made. For example, DNA PfPol δ and PfPCNA both show similar accumulation patterns of protein and mRNA but appear to have distinct mechanisms regulating their temporal expression.

Horrocks *et al.*, (1996) have found that both of these proteins accumulate principally during the trophozoite and schizont stages, but their transcripts are confined to the trophozoite stage. An assessment of promoter activity however, shows that the PfPCNA promoter is active throughout all of the intraerythrocytic stages, whereas PfPol δ promoter activity is confined to the trophozoite/schizont stages. These findings suggest that in the case of PfPCNA, transcript levels are regulated post-transcriptionally, in contrast with PfPol δ where transcript levels and promoter activity are interrelated.

Similar data have also been obtained for PfTopoisomerase I where promoter activity, transcript accumulation and protein expression all follow a similar pattern to PfPol δ . The relationship between protein accumulation and enzyme activity was also investigated, and although levels of PfTopoI were found to be similar in trophozoite and schizont

stage parasites, enzyme activity was found to increase markedly in schizonts. These findings could indicate that the protein undergoes post-translational modification(s) as the parasite progresses from trophozoite to schizont stages (Dr. K. Tosh -in preparation).

DNA replication in the malaria parasite takes place at five distinct points during its complex life cycle in the human host and mosquito vector (White and Kilbey 1996). Discussion will be limited to DNA replication in the parasite that occurs during the blood stages within the human host. Here, DNA replication first starts in the trophozoite and continues into the schizont stage. Up to four rounds of nuclear division result in a schizont with up to sixteen nuclei (Tilney and Tilney 1996). Cytokinesis then gives rise to merozoites which are able to invade more erythrocytes after the schizont bursts. Thus it seems likely that the temporal regulation of PfPCNA and PfPol δ in particular, and possibly PfTopoI as well, during the trophozoite and schizont stages may be intimately linked to the onset of DNA replication and nuclear division.

The relationship between DNA replication in the parasite and the pattern of expression of PfTopoisomerase II has been investigated and the results of this study will be presented here. Part of the work (measurement of promoter activity) was done in collaboration with Dr. Paul Horrocks.

The gene encoding PfTopoII from the parasite was isolated and characterised in 1994. The 4191 bp open reading frame (revised from 4194 bp, Cheesman *et al.*, 1994) contains no introns. Sequences conserved in topoisomerase II from other species are present in the *falciparum* sequence, but two unique tracks encoding asparagine repeats are predicted to fall towards the N-terminal domain of the protein. PfTOP2 is a single-copy gene encoding a protein of 1397 amino acids (160 kDa/subunit). The gene shares 47% sequence identity with the human TopoII α homologue. Northern analysis reveals a transcript of approximately 5.8kb and the gene has been localised to chromosome 14. Preliminary qualitative data indicates that the protein is detectable at all stages of intraerythrocytic development.

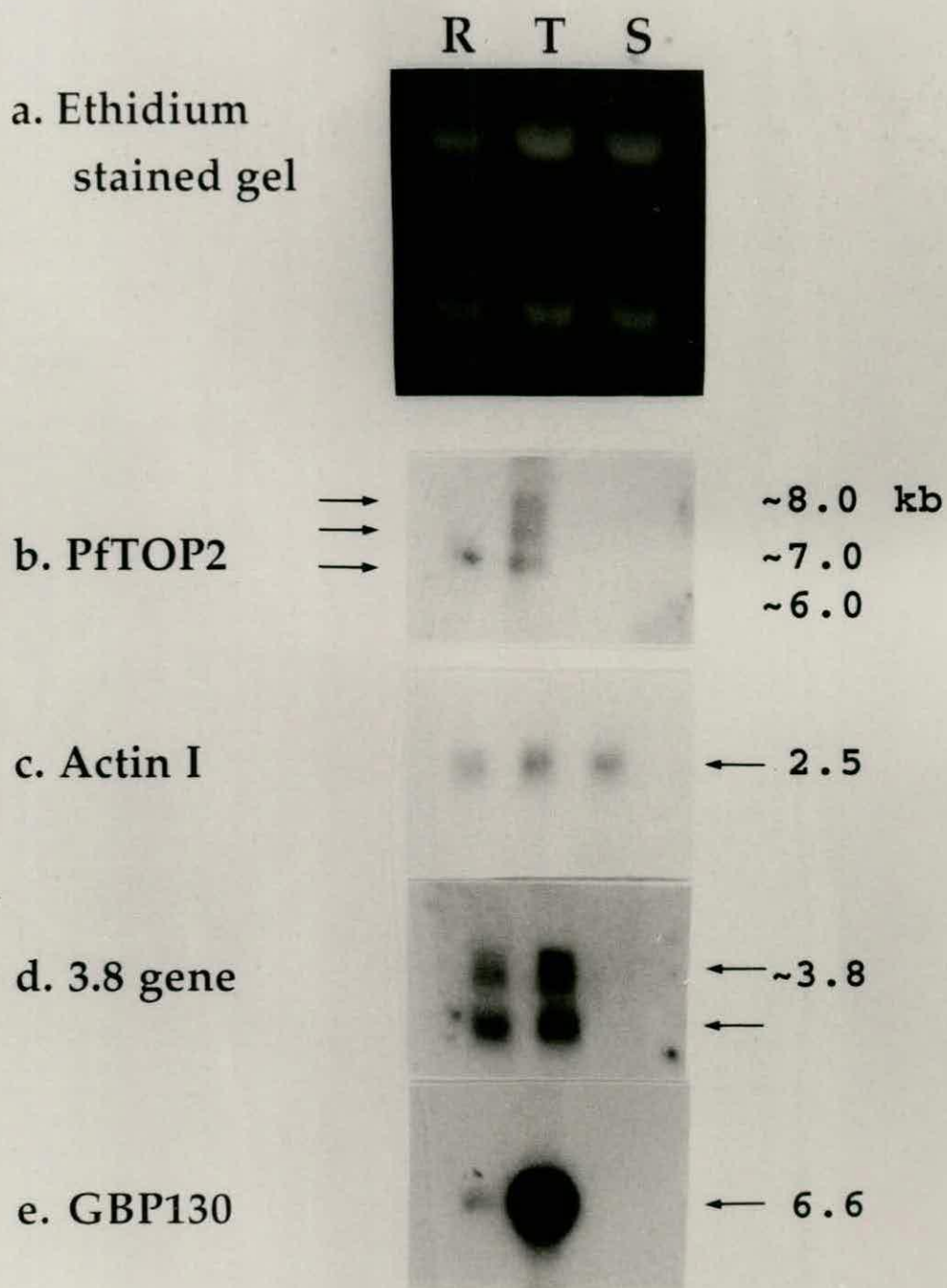
4.2 The PfTOP2 message is temporally regulated and peaks in trophozoites.

Intraerythrocytic stage parasite cultures were synchronised using sorbitol treatment and harvested at the ring, trophozoite or schizont stage as described in section (2.7.3). The degree of synchrony was checked by microscopic analysis of Giemsa stained parasites (2.7.2). 10µg of RNA from each stage prepared by method (2.4.1) was size fractionated on a formaldehyde gel alongside RNA size standards (Gibco). The gel was blotted to Genescreen Plus membrane and probed with gene-specific DNA sequences (figure 1). Three bands appear when the blot is probed with the random-labelled 1.8kb 5' *KpnI/HindIII* fragment of the PfTOP2 gene, corresponding to nucleotides 1-1795. The most prominent of these is at about 6.0 kb. However, two other bands at approximately 7.0 and 8.0 kb are also recognised by this probe. These are DNaseI resistant. The bands appear in trophozoites, but they are absent from schizont preparations. Small amounts of the smallest of the three transcripts appear in rings.

Microscopic examination of the samples revealed around 7% contamination of the ring stage RNA by trophozoites and schizonts, whilst the trophozoite sample contained about 16% contamination with rings. Therefore, in order to confirm the pattern of transcript accumulation, a series of probes derived from genes with well known stage-specific patterns of expression were used to probe the same northern blot. The 3.8 gene probe (Lanzer *et al.*, 1992), which is specific for rings and trophozoites only, behaves in the expected manner. Whilst GBP 130, a trophozoite-specific probe (Lanzer *et al.*, 1992) was found to hybridise predominantly to trophozoite stage RNA as expected, although prolonged exposure reveals a faint signal in the ring-stage sample. The actin I probe (Wesseling *et al.*, 1989) gave rise to signals of almost equal intensity in rings, trophozoites and schizont stage RNA, in line with its known specificity. This result also helps to confirm that almost equal amounts of RNA were loaded in the lanes. Taken together, these data suggest that the PfTOP2 messages accumulate principally in trophozoites but are absent from schizonts and possibly present at low levels in rings.

Figure 1.

Northern blot analysis of total RNA extracted from parasites synchronised in intraerythrocytic stages. The proportion of rings (R), trophozoites (T) and schizonts (S) in each sample was as follows; Ring sample (93R:4T:3S); trophozoites (16R:84T:0S); schizonts (0R:0T:100S). The positions and sizes of the three PfTOP2 transcripts are indicated. Panel (a) shows the 18S and 28S ribosomal RNA bands present in the RNA stained with ethidium bromide.



4.3 PfTopoII levels are highest in trophozoite and schizont stage parasites.

The antiserum raised against the C-terminal of PfTopoII (chapter 3) was used to probe western blots (8% SDS-PAGE) of equivalent numbers of size fractionated parasites synchronised in ring, trophozoite or schizont blood stages (figure 2). Three major bands of approximately 160/175 kDa in size were recognised in trophozoite and schizont samples only. It is not known whether the lower two bands seen on these westerns are proteolytic fragments of the larger protein, or alternatively, the products of differential processing events occurring *in vivo*; for example different phosphorylation states of the protein.

Figure 2 also shows that similar levels of protein are detected by the antisera in trophozoite and schizont stage parasites. Far less recognisable protein is present in rings however, and interestingly only a single band, the largest of the three, is present in detectable amounts.

4.4 PfTopoII activity is highest during schizogony.

Crithidia fasciculata kinetoplast DNA (KDNA) consists of a network of mini and maxicircles. The minicircles are highly concatenated, a feature which can be exploited as a means of testing for the decatenation activity of a type II topoisomerase. Decatenation results in the formation of both open circular (OC) and covalently closed circular (CCC) DNA species which can be separated from each other by gel electrophoresis and from the unresolved catenated material which remains in the well.

Parasites were harvested as described in section 2.7.4. The degree of synchrony was determined by Giemsa staining and microscopic analysis (2.7.2). Parasite pellets were quick frozen and lysed by the addition of topoII assay buffer (2.9.1).

The decatenation activity of protein extracts from equal numbers of parasites at each intraerythrocytic developmental stage was tested (2.9.1) and the results of a typical experiment are shown in figure 3. There is little evidence of decatenation in rings. Virtually all of the input KDNA is retained in the well and there are only traces of OC and CCC DNA. In contrast, decatenation is readily detected in the extracts from trophozoites and schizonts, with higher activity in the latter as evidenced by the greatly

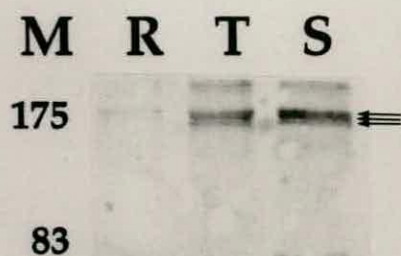
Figure 2 .

Western blot analysis of protein prepared from parasites synchronised in intraerythrocytic stages. The proportion of rings (R), trophozoites (T) and schizonts (S) in each sample was as follows; Ring sample (88R:12T:0S); trophozoites (5R:87T:8S); schizonts (3R:26T:71S). M. represents the positions of the prestained protein markers (NEB). Sizes indicated are in kilodaltons.

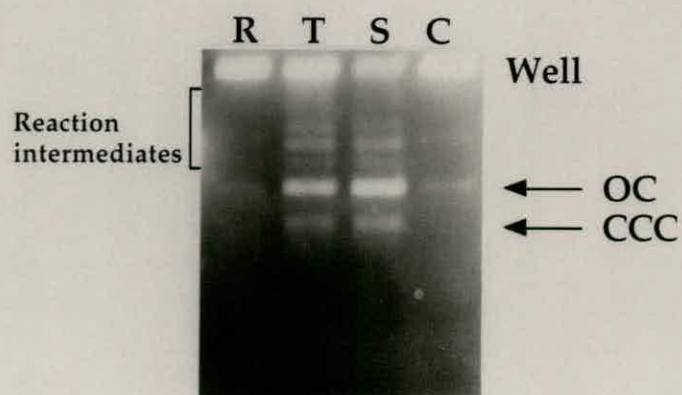
Figure 3.

Decatenation activity assay using KDNA as substrate with parasite extract from asexual parasites synchronised in ring, trophozoite and schizont stages. The proportion of rings (R), trophozoites (T) and schizonts (S) in each sample was as follows; Ring sample (82R:10T:8S); trophozoites (18R:75T:7S); schizonts (36R:5T:60S); C. negative control sample without parasite extract. The positions of open circular DNA (OC) and covalently closed circular DNA (CCC) reaction products are indicated.

2



3



130 (a)

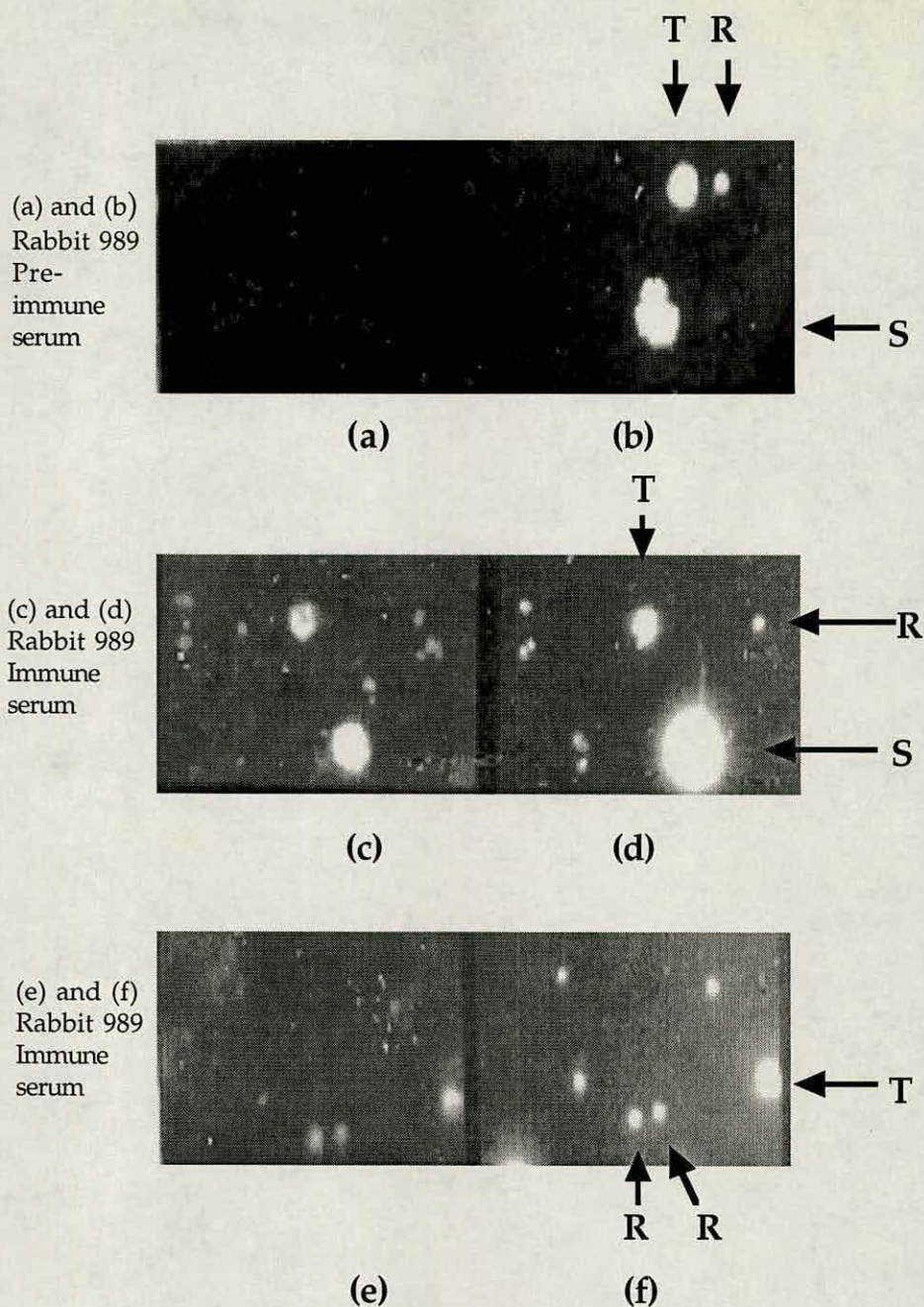


Figure 4. Indirect immunofluorescence assay of *P. falciparum* blood stage parasites. (R) Ring stage parasite. (T) Trophozoite stage parasite. (S) Schizont stage parasite.

Thin blood smears from unsynchronised populations of parasites were acetone fixed and PfTopoII cross-reactive parasites were detected using anti-rabbit IgG RITC conjugated second antibody. Parasite nuclei were counter-stained with DAPI (panels b, d and f) and rhodamine (panels a, c and e).

reduced amounts of unresolved KDNA in this sample.

4.5. PfTopoII protein can be detected in all blood-stage parasites.

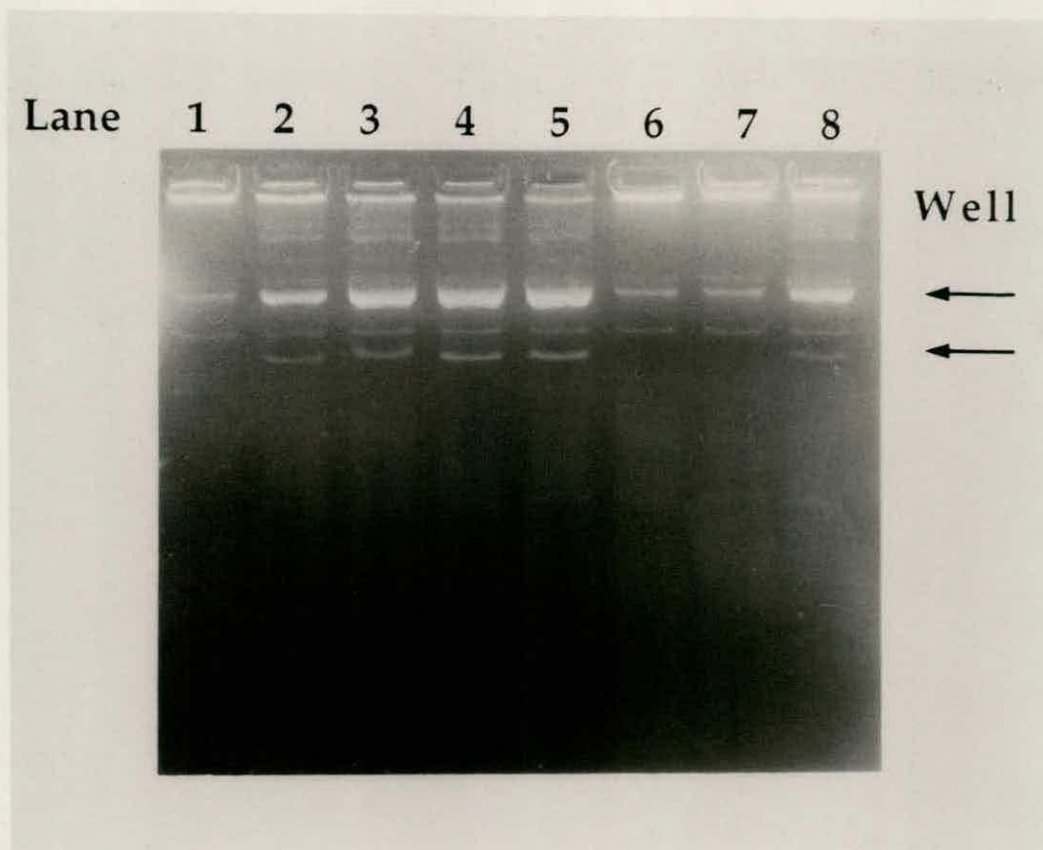
Figure 4 shows an immunofluorescence assay conducted on unsynchronised populations of intraerythrocytic parasites. Pre-immune and immune sera obtained from rabbit 989 was used to probe the parasites which were counter-stained with DAPI (2.5.7). Figure 4 shows that trophozoite and schizont stage parasites cross-react most strongly with the immune sera. Ring stage parasites also cross-react with the immune sera, but to a lesser extent. The pre-immune sera failed to cross-react with any intraerythrocytic parasite stage. This result corroborates the western blot analysis data shown in Figure 2 that indicated that PfTopoII levels are far lower in ring stage parasites.

4.6 PfTopoII activity can be immunodepleted.

The same antiserum was also tested for its ability to inhibit the decatenation activity of parasite extracts. As can be seen in figure 5, the decatenation activity derived from unsynchronised populations of parasites was completely inhibited following exposure to antiserum at dilutions of 1:100 and 1:500; with activity reappearing at 1:2000. Pre-immune serum from the same rabbit had no inhibitory effect at the same dilutions. The same antiserum however, failed to inhibit the decatenation activity of purified topoisomerase II α at 1:100 dilution (data not shown).

Figure 5

Decatenation activity assay of immunodepleted parasite extract from unsynchronised parasites using KDNA as substrate. Lane 1, negative control; KDNA without parasite extract. Lane 2, positive control KDNA with parasite extract. Lanes 3-5, parasite extract with pre-immune serum diluted at 1:100, 1:500 and 1:2000 respectively. Lanes 6-8, parasite extract with immune serum diluted at 1:100, 1:500 and 1:2000 respectively. The lower arrow indicates the position of the covalently closed circular reaction product (CCC) and the upper arrow indicates the position of the open circular reaction product (OC).



4.7 Discussion.

An analysis of the stage-specific expression of the gene encoding topoisomerase II of the human malarial parasite, *P. falciparum*, during the intraerythrocytic developmental stages has been conducted. Nuclear run-on analysis (conducted by Dr. Paul Horrocks) indicates that the PfTOP2 promoter is active at low levels in the stages which follow upon invasion of the red blood cell, but that activity increases markedly as the parasite progresses into the latter stages of development (trophozoites and schizonts). In this respect its pattern of activity is intermediate between the promoters of PfDNAPol δ and PfTopoI which are both inactive in rings but active in trophozoites/schizonts stages. This is in contrast to PfPCNA, the promoter activity of which is similar in rings and trophozoites/schizont stage parasites (Horrocks *et al.*, 1996).

The steady state accumulation of PfTOP2 transcripts reflects the pattern of promoter activity, but the picture is complicated by the presence of three distinct transcripts at approximately 6, 7 and 8kb. The major 6kb PfTOP2 transcript alone, is detectable in ring stage parasites at very low levels. All three transcripts are present in trophozoites, but all are completely absent from schizont-derived RNA. This absence is not attributable to a failure of the RNA to transfer to the membrane from the gel: the behaviour of the Actin I probe suggests that approximately equal amounts of RNA have been loaded for each lane and successfully transferred. Multiple transcripts of *P. falciparum* are not unknown, indeed the PfPCNA gene encodes two transcripts of 1.85 and 2.2 kb (Horrocks *et al.*, 1996). The 3.8 gene also expresses two polymorphic messages (Lanzer *et al.*, 1992).

This raises interesting questions concerning where transcription initiates and terminates in the PfTOP2 gene. Time constraints have precluded a study of the untranslated flanking regions of this gene, however.

DNA synthesis in the asexual parasite starts at the trophozoite stage, the same stage at which we have shown that peak accumulation of PfTOP2 transcripts also occur. Similar findings have been reported for *Drosophila*, where, during early embryogenesis and pupation, stages which have a high mitotic index, TopoII transcript production also increases (Fairman and Brutlag 1988).

The relative quantities of enzyme have also been determined immunologically, and again, low levels of protein (on a per parasite basis) are present in rings, but increasing amounts appear in trophozoites and schizonts. A triplet of bands (160/175 kDa) was detected by the antiserum on western blots of size fractionated trophozoite and schizont extracts, but only the largest of the bands is unambiguously detectable in the ring stage parasite extracts. Triplets of bands have also been reported in topoisomerase II westerns derived from other species. In *Drosophila*, for example, bands of 132, 145 and 166 kDa were reported to coincide with the onset of early embryogenesis and pupation (Heller *et al.*, 1985). In addition, Sander and Hsieh (1983) reported that all three of these polypeptides were active. It is possible that the two smaller bands represent partially degraded forms of the full length protein or may be the products of differential post-translation modification. What is possibly surprising is that the levels of immunologically detectable PfTopoII persist into schizogony after the mRNA has ceased to be detectable. A similar situation pertains to the major DNA polymerase, PfDNA Pol δ . It appears that several of the replication proteins are produced in sufficient quantities at the trophozoite stage, and are stable enough, to satisfy the requirements of DNA replication during schizogony without being renewed.

Decatenation assays, which are a definitive test for type II topoisomerase activity, have shown that activity increases as the parasite progresses from the trophozoite into the schizont developmental stage, although there appears to be very little difference in protein levels. Post-translational modifications are known to play a role in the control of topoisomerase II activity and phosphorylation by casein kinase II specifically targets the species-divergent carboxyterminal domain of the enzyme in yeast (Cardenas *et al.*, 1992). Phosphorylation acts as a regulator of many eukaryotic gene functions and it has also been shown to enhance the topoisomerase relaxation activity in *Drosophila* (Ackerman *et al.*, 1985). In contrast, poly-ADP-ribosylation appears to be an inhibitor of topoisomerase II in a number of species (Darby *et al.*, 1985 and Scovassi *et al.*, 1993). Further study of PfTopoII will be required to determine whether

the enzyme is subjected to similar post-translational modifications.

The same polyclonal antiserum was also found to be proficient at inhibiting the decatenation activity of parasite extracts; a property not shared by pre-immune serum derived from the same rabbit. In contrast, the decatenation activity of human TopoII α , following treatment with the same antiserum, was completely unaffected (data not shown). This result corroborates previous evidence (western blot and IFA data) concerning the specificity of the antiserum.

Chapter 5

Expression of PfTopoisomerase II in baculovirus-infected insect cells.

5.1 Introduction.

There are several potential advantages to choosing baculovirus as a means to express foreign proteins. Firstly, the baculovirus expression system is tailored for high level expression of eukaryotic genes and large open reading frames are easily accommodated. Because gene expression occurs within an insect cell environment, most of the common post-translational modifications to proteins that are known to occur in eukaryotes appear to be carried out in this system (eg. phosphorylation, signal peptide cleavage and glycosylation). This is an advantage over prokaryotic expression systems which may not make the appropriate modifications to eukaryotic gene products (O'Reilly 1992).

The circumsporozoite protein (amino acids 1-412) of *P. falciparum* and the major ookinete surface protein of *Plasmodium berghei* (Pbs21) have both been expressed in baculovirus (Jacobs *et al.*, 1991 and Matsuoka *et al.*, 1996) and more recently, falcipain, a cysteine protease involved in haemoglobin degradation, has also been functionally expressed (Salas *et al.*, 1995) demonstrating the utility of the system in the case of *P. falciparum* proteins. Full length PfPCNA has also been successfully expressed in our laboratory (White, J.W.-unpublished results). In addition, expression of human topoisomerase I in baculovirus gave rise to a 100-fold increase in expression compared to human cell lines, and the recombinant protein was found to be biochemically indistinguishable from the native enzyme (Zhelkovsky and Moore 1994) indicating that the system may be useful for the functional expression of topoisomerases.

Background to the baculovirus expression system

Baculoviruses (family Baculoviridae) belong to a diverse group of DNA viruses that infect many different species of insect. They are however, highly species specific and can only infect their natural host. *Autographica californica* nuclear polyhedrin virus (Ac.NPV) infects *Spodoptera*

frugiperda (fall army worm) insect cells.

Progress of the infection in cell culture.

The infection cycle follows three distinct phases: (1) early, (2) late and, (3) the very late phases. Briefly these correspond to (1) reprogramming the cell for virus replication, (2) production of budded virus particles and, (3) production of occluded viruses.

In phase 1 (0-6 hours) the budded form of the virus enters the insect cell by adsorptive endocytosis and migrates into the nucleus. Once inside the nucleus, the viral core appears to be released from the "capped" end of the capsid. Viral RNA can be detected within 30 minutes of the virus being adsorbed into the insect cell. This indicates that movement of viral DNA to the nucleus and the initiation of viral transcription are rapid processes. Cytoskeletal rearrangements, cytoplasmic, and nuclear changes also occur during this phase which appears to be mediated by the components of the viral inoculum and the viral proteins expressed from early viral genes.

The late phase of the infection (phase 2) starts around 6 hours post infection (p.i) and lasts up to 20-24 hours. This phase involves a period of intense viral DNA replication, late gene expression, and budded virus production. Progeny nucleocapsids leave the nucleus, possibly by a variety of routes and travel through the cytoplasm to the cytoplasmic membrane where they bud off individually.

The very late phase (Phase 3), starts around 20-24 hours (p.i). Virus particles start to become embedded in a homogenous matrix of polyhedrin protein in the insect cell nucleus during this time. These viruses are called occluded virus particles (OV) and are released upon cell lysis during the late phase of the cycle. The polyhedrin protein is essential for the transmission of the virus, but expression of the protein is not necessary for virus survival under cell culture conditions. The OV bodies protect the virus from environmental inactivation (for example desiccation) prior to transmission to the insect larvae which become infected when they consume the OV during feeding. The virus is released under the alkaline conditions of the insect gut when ingested.

The Baculogold system (PharMingen) uses a baculovirus containing a lethal deletion which can be rescued when it recombines homologously

with a transfer vector containing the foreign gene when the virus and the transfer vector are present together inside the insect cell. Expression of the gene of interest is driven by a strong viral promoter [eg. the polyhedrin promoter, (polh)]. Inactivation of the polh gene by deletion or insertional activation of a foreign gene gives an occlusion body negative phenotype (occ-). This phenotype is readily distinguishable from the wild type occ+ baculovirus under the microscope and thus provides a marker with which to select recombinant baculoviruses.

The polh promoter was used to drive expression of the PfTOP2 gene. It and was chosen not only because of the ease of selection for recombinant viruses, but also because it is expressed very late in the infection (16/18 hours p.i.) when host cell gene expression has been shut down. In addition, this promoter has been reported in some instances to drive expression of foreign proteins to levels of up to 20-50% of total cell protein.

5.2 Engineering the PfTOP2 gene for expression in baculovirus.

The open reading frame of the PfTOP2 gene consists of five overlapping genomic clones (figure 1). In order to obtain one continuous open reading frame that had unique 5' and 3' restriction sites compatible with the baculovirus transfer vector it was necessary to re-engineer the gene .

For some baculovirus genes, the context of the AUG translation initiation codon appears to be important for expression. For example, an A is often found in the -3 position and pyrimidines often appear at positions -1 and +5. Table 1 summarises this. Also the absence of a G in the seven nucleotides immediately preceding the AUG appears to be preferred. Although it is recognised that these rules may not be absolute, they do provide useful guidelines (O'Reilly 1992).

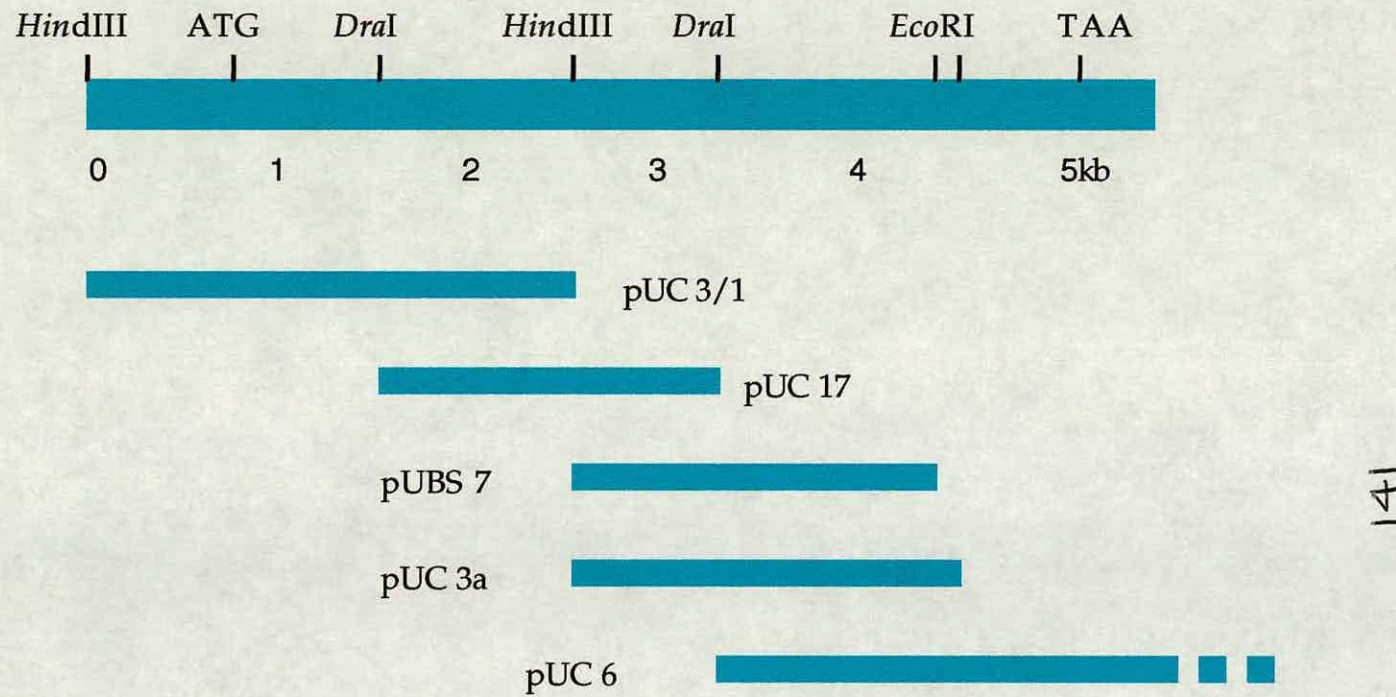


Figure 1. Diagrammatic representation of the PFTOP2 gene with selected restriction sites.

Table 1. The AUG context of the baculovirus polh and p10 genes.

<i>Gene</i>		<i>AUG context</i>		
polh		CCUAUAAAU AUG CCGG		
p10		UUUACAAU CAUG UCAA		
	Consensus	A	Y	Y
	Position	-3	-1	+5

Taking these factors into account, the context of the AUG was changed to incorporate these features into the 5' oligonucleotide [Figure 3 (a and b)].

It was necessary to construct the PfTOP2 ORF using two different vectors (pUC19 and pUBS1) because two *Pst*I and *Bam*HI sites are present in the gene.

The ATG to *Hind*III fragment of the PfTOP2 gene (figure 2) was constructed as follows. The 5' genomic clone pUC3/1 ([figure 1] Cheesman *et al.*, 1994), was used as a template for a PCR using the oligonucleotides G6166/G6165. A PCR product of 1030 bp was isolated, purified and restricted with *Kpn*I and *Pst*I and ligated into pUC19 (Yanisch-Perron *et al.*, 1985) containing the *Pst*I/ *Hind*III fragment of the gene.

The remainder of the gene encompassing the *Hind*III to TAA fragment (figure 2) was constructed by restricting the genomic clone pUBS 7 ([figure 1] Cheesman *et al.*, 1994) with *Hind*III and *Bam*HI and subcloning the fragment into vector pUBS1. The distal *Bam*HI to TAA fragment was made using pUC 6 ([figure 1] Cheesman *et al.*, 1994) as a PCR template to amplify this region using the oligonucleotides 445Y/G5626. A 1376 bp PCR fragment was isolated, purified and restricted with *Bam*HI and *Sac*I, then cloned into pUBS containing the *Hind*III/*Bam*HI fragment.

The complete *Kpn*I/*Sac*I fragment (figure 2) was restricted with *Bgl*II which cuts just inside the *Kpn*I and *Sac*I sites to remove the entire ORF which was subcloned into the baculovirus transfer vector pVL1393 figures 4 and 5). Recombinant plasmids were screened to ensure correct orientation of the gene relative to the promoter, and cloning borders were sequenced.

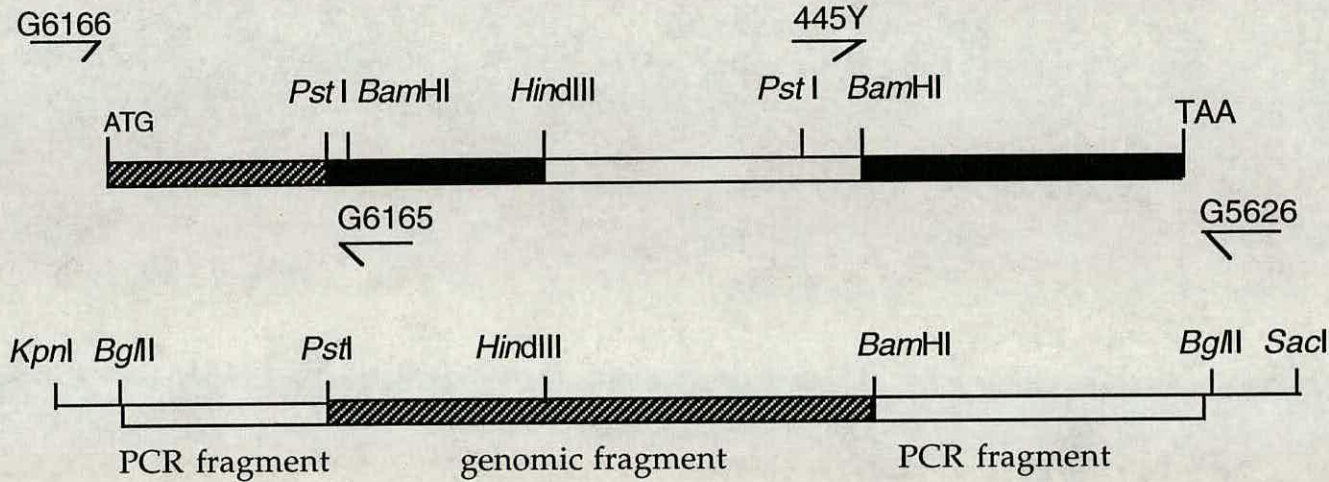


Figure 2. Strategy used to engineer the five genomic PftOP2 clones into one continuous open reading frame for expression in baculovirus. Top diagram represents the PftOP2 gene and shows the positions of the amplifying oligonucleotides G6166/G6165 and 445Y/G5626. Bottom diagram represents the modified PftOP2 open reading frame in pUBS 1.

The 5' *KpnI*/*PstI* fragment was made by PCR amplification using oligonucleotides G6166 and G6165. The 3' *BamHI*/*SacI* fragment was made by PCR amplification of oligonucleotides 445Y and G5626. Both fragments were sequenced to ensure that no PCR introduced mutations had arisen. The 5' *KpnI*/*PstI* fragment was restricted and subcloned into pUBS 1 containing the *PstI*/*HindIII* genomic fragment. The 3' *BamHI*/*SacI* fragment was restricted and subcloned into pUC19 containing the *HindIII*/*BamHI* genomic fragment. The *HindIII*/*SacI* fragment was restricted and subcloned into pUBS 1 containing the *KpnI*/*HindIII* fragment. The complete gene cassette was excised as a *BglII* fragment and subcloned into the baculovirus transfer vector pVL1393 (figures 4 and 5) for expression.

(a)

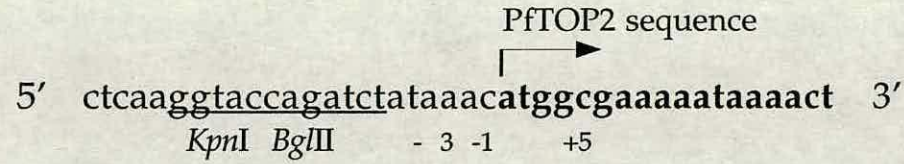


Figure 3 (a) Sequence of the 5' oligonucleotide G6166 that was used to amplify and introduce unique restriction sites into the 5' end of the gene. Sense PfTOP2 nucleotides are shown in bold. The adenosine at position -3 and the cytosine at -1 were incorporated to conform to the baculovirus polh and p10 AUG consensus sequence.

(b)



Figure 3 (b) Sequence of the 3' oligonucleotide G5626 that was used to amplify and introduce unique restriction sites into the 3' end of the PfTOP2 gene.

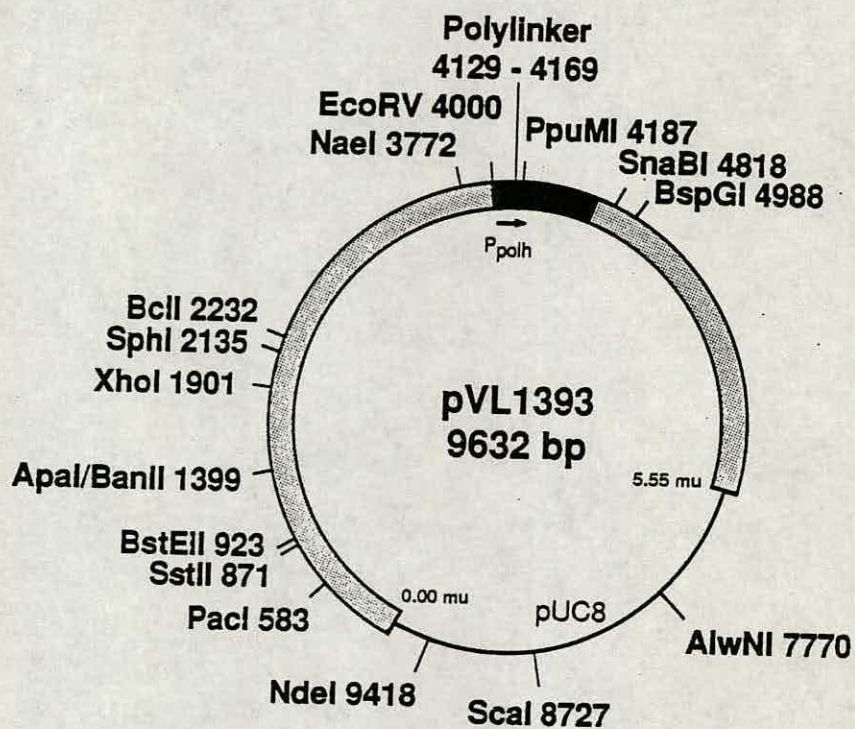
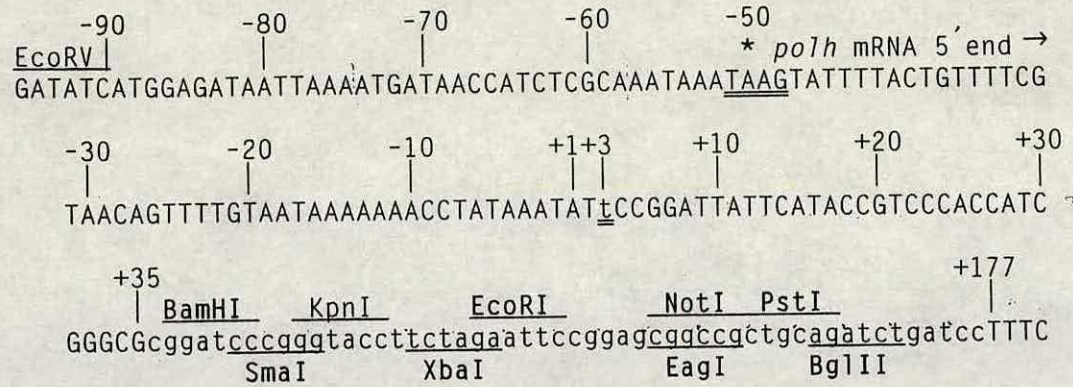


Figure 4. Plasmid map of the baculovirus transfer vector pVL1393. The positions of the polh promoter and polylinker are indicated (Source-Invitrogen).

pVL1393



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Figure 5. Diagrammatic representation of the baculovirus transfer vector pVL1393 polylinker.

The *polh* ATG is mutated to ATT, and the polylinker is located at position +35. The plasmid was designed this way because it was thought that sequences downstream from the *polh* ATG might contribute to optimal promoter activity (O'Reilly 1992).

A brief summary of the steps involved in making the PfTOP2 ORF is shown in figure 2.

5.3 Identification of recombinant baculoviruses from co-transfection of insect cells.

5.3.1 Selection of candidate recombinant baculoviruses.

The recombinant baculovirus transfer vector pVL1393 harbouring the PfTOP2 gene was used in conjunction with linearised BaculoGold™ DNA to co-transfect *S. frugiperda* (Sf9) cells (2.3.10.1). Wild type AcNPV was also used to infect a parallel batch of Sf9 cells to provide an occ⁺ phenotype positive control. Sf9 cells were also seeded without virus to provide a negative control which could be used for comparison. Following incubation at 27°C for four days, co-transfected Sf9 cells were examined for signs of infection. Microscopic analysis revealed that the cells were enlarged, exhibited the occ⁻ phenotype and many were floating freely in the medium; these signs are characteristic of baculoviral infection. Wild type infected cells also appeared enlarged but showed the occ⁺ phenotype. Supernatants from the co-transfection and wild type infection were harvested and stored at 4°C.

Pure viral stocks were made from both viral sources as outlined in section 2.3.10.2. This involved infecting Sf9 cells with viruses diluted at 10⁻⁵ to 10⁻⁸ in insect cell medium and overlaying the infected cells with an agarose plaquing solution. After four days incubation distinct plaques were visible following staining of the agarose with neutral red dye. Six individual, well isolated candidate recombinant PfTOP2 baculovirus plaques were picked and 4 were used for amplifying the virus to an acceptably high titre stock (2.3.10.3). Amplification of the viruses gave titres in the order of around 5 X 10⁷ to 5 X 10⁸ pfu/ml.

5.3.2 Identification of recombinant baculoviruses by Southern blot analysis.

Total recombinant virus-infected insect cell DNA was prepared from 2 viruses (1A and 2A) together with a AcNPV and uninfected Sf9 cell control. These were restricted with *Bgl*II, Southern blotted (2.3.4) and

hybridised to a PftOP2 specific oligonucleotide to establish whether any of the candidate viruses were harbouring the PftOP2 gene. The results of the Southern blot analysis are shown in figure 6 which demonstrates that only candidate recombinant baculovirus DNA hybridises to the probe. No cross-hybridisation of the probe to DNA derived from the uninfected or AcNPV infected cells was observed.

5.4 Primary characterisation of recombinant PftOP2 baculovirus expression.

In order to monitor expression of the PftOP2 gene, Sf9 cells were infected for variable periods of time (0-72 hours p.i.) with recombinant baculoviruses 1A and 2A. Total protein synthesis was monitored by preparing whole cell extracts (2.5.4.3) that were analysed by SDS-PAGE fractionation and Coomassie blue staining. Using this method of detection, no evidence of gene expression was observed, however.

Western blot analysis of whole cell extracts was performed to establish whether any recombinant PftTopoII was being produced. This was done in an attempt to increase the sensitivity and specificity of detection of the recombinant protein, in the event that it was being produced in very small quantities. Polyclonal antiserum raised against the N-terminal domain of PftTopoII as a GST fusion protein (Cheesman *et al.*, 1994) was used for analysis of whole cell extracts size fractionated by SDS-PAGE and western blotted to nitrocellulose. This antiserum did not cross-react with proteins derived from recombinant or wild type virus infected cells, however. At this point the polyclonal antiserum raised against the carboxyterminal region of PftTopoII that was discussed in Chapter 3 had not been made.

5.5 Northern blot analysis of recombinant baculoviruses.

Northern analysis of recombinant baculoviruses was used as a means to establish whether the PftOP2 gene was being transcribed by the polh promoter *in vivo* as there were no obvious signs of protein expression.

20 µg of total RNA extracted from insect cells infected with wild type or recombinant PftOP2 baculoviruses 1A and 2A for periods of 24 and 48

Figure 6.

Southern blot of total DNA isolated from Sf9 insect cells. The DNA was restricted with *Bgl*III, size fractionated on a 1% agarose gel, and blotted to Genescreen Plus membrane. The PfTOP2 positive control was derived from a restriction digest of the PfTOP2 gene in transfer vector pVL1393. The prehybridised membrane was probed using a PfTOP2 specific oligonucleotide (856S).

Lane 1. Genomic DNA isolated from uninfected Sf9 insect cells. Lane 2. Total DNA isolated from wild type virus (AcNPV) infected Sf9 cells (negative control). Lane 3. PfTOP2 gene (positive control). Lane 4. Total DNA isolated from recombinant baculovirus 1A infected Sf9 cells. Lane 5. Total DNA isolated from recombinant baculovirus 2A infected Sf9 cells. The positions of the *Bgl*III fragments are indicated.

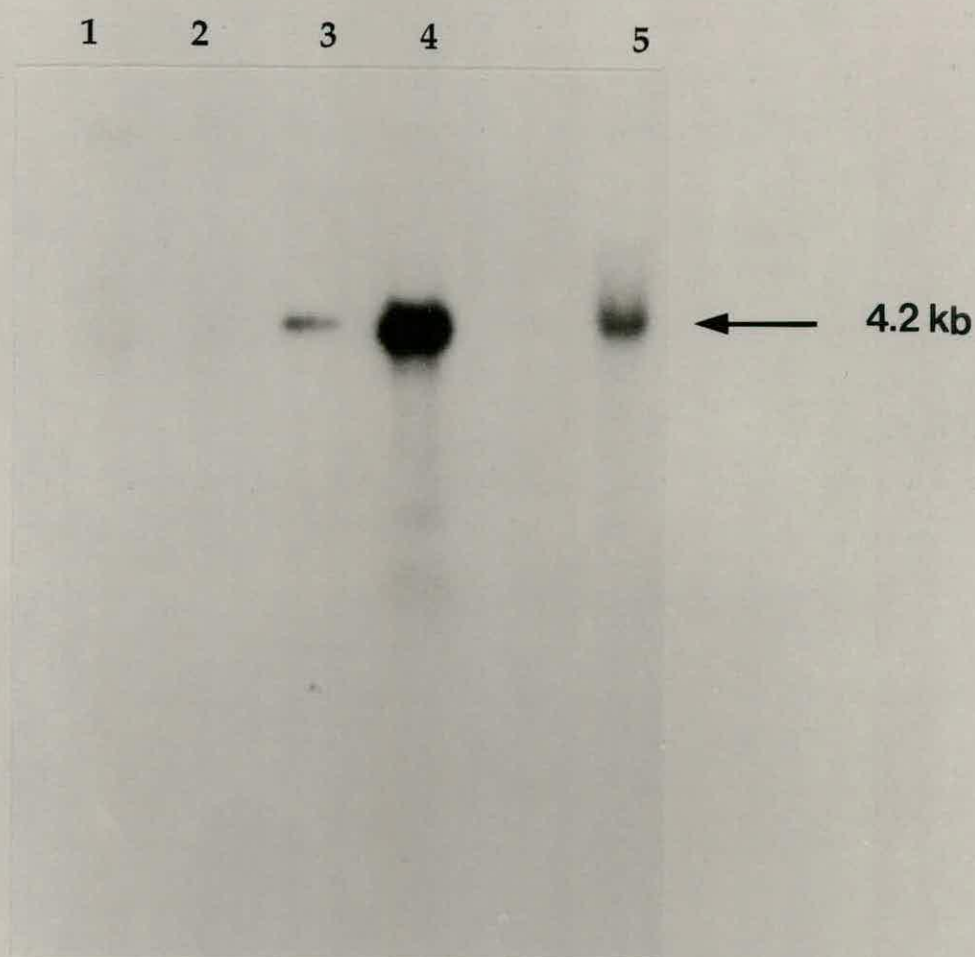


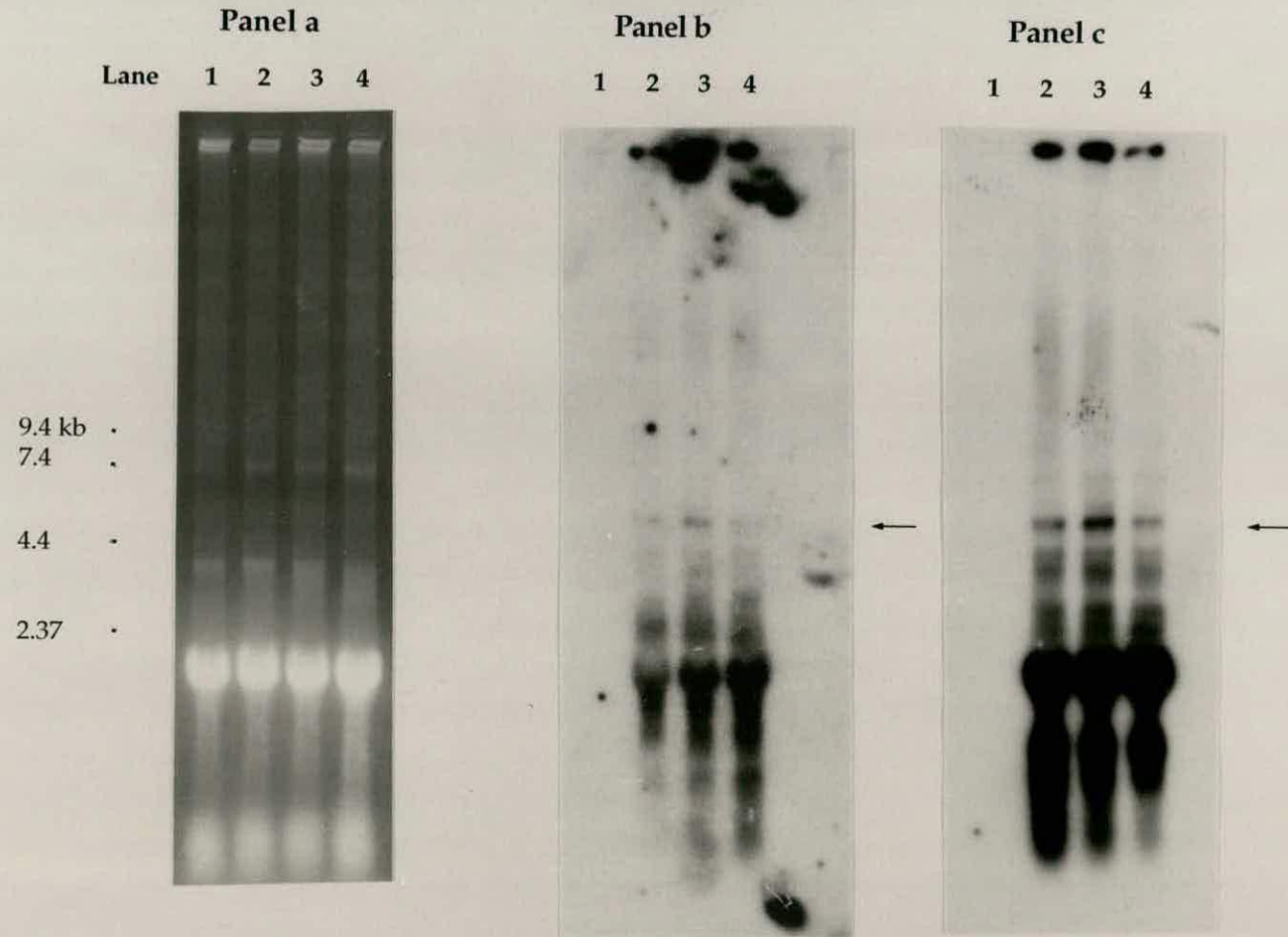
Figure 7.

Northern blot analysis of total RNA extracted from insect cells harboring recombinant PfTOP2 or wild type baculovirus.

Panel a. Ethidium bromide stained formaldehyde gel prior to northern blotting. Lane 1; wild type baculovirus at 24 hours post-infection (p.i.). Lane 2; recombinant PfTOP2 baculovirus 1A at 24 hours p.i. Lane 3; recombinant PfTOP2 baculovirus 2A at 24 hours p.i. Lane 4; recombinant PfTOP2 baculovirus 2A at 48 hours p.i.

Panel b. Northern blot of the ethidium bromide stained formaldehyde gel shown in Panel a, probed with the 5' *KpnI/PstI* 1030 bp fragment of the PfTOP2 gene. The position of the 5 kb transcript is indicated.

Panel c. The same northern blot as shown in Panel b, after stripping and reprobing with the 3' *BamHI/SacI* 1376 bp fragment of the PfTOP2 gene. The position of the 5 kb transcript is indicated.
The positions of the RNA size standards are indicated in kilobases.



hours as described in section 2.4.3 was size fractionated and blotted to a Genescreen Plus membrane. The northern blot was hybridised to the ^{32}P random-labelled 1030 bp 5' *KpnI/PstI* fragment of the PfTOP2 gene (figure 2), unbound probe was removed by washing and the membrane exposed to film.

No hybridisation of this probe to the wild type baculovirus RNA was observed (figure 7 b-lane 1). However, a prominent band of approximately 5 kb was detected by this probe in all recombinant baculoviral RNA preparations (figure 7 b - lanes 2, 3 and 4). It is probable that the 5 kb signal represents the full length recombinant PfTOP2 mRNA as its size is consistent with that anticipated from the ORF of the gene (4191 bp). Several other highly abundant lower molecular weight polymorphic transcripts are also recognised by this probe but only in recombinant preparations. The same northern blot was stripped and exposed to film to ensure that all bound probe was removed. The blot was then reprobed with the ^{32}P random-labelled 3' end of the PfTOP2 gene (1376 bp *BamHI/SacI* fragment- see figure 2) This probe also failed to hybridise to wild type baculovirus mRNA, but recognised the same 5 kb transcript in all of the recombinant RNA preparations. A similar pattern of lower molecular weight abundant polymorphic transcripts were also seen to hybridise to this 3' probe (figure 7 c).

Figure 7 (a) shows the ethidium bromide stained formaldehyde gel prior to northern blotting. There is some evidence of DNA contamination in the samples, as testified by the presence of ethidium bromide staining material in the wells. Additionally there is also some "smearing" of the RNA samples, and the ratios of the 28S and 18S ribosomal RNA bands are unequal, suggesting that there may be some RNA degradation. In order to determine whether this "smearing" effect could be the result of DNA contamination of the samples, they were DNase I treated. DNase I treatment decreased the level of ethidium stained material observed in the wells (result not shown). UV spectrophotometry of the RNA samples showed that the quantity and quality of each of the samples were within normal limits. The $\text{OD}_{260}/\text{OD}_{280}$ ratio were between 1.9 - 2.0 suggesting that the samples were not contaminated with residual phenol or protein.

In addition the OD₂₃₀, which is a measure of salt contamination was also within normal limits.

5.6 Time course analysis of protein production from a recombinant PfTOP2 baculovirus.

Western blot analysis (7% SDS polyacrylamide gel) of total protein from whole cell extracts (equal numbers of cells) infected with recombinant baculovirus 1A at time points of 18, 20, 22 and 24 hours was conducted (figure 8). This time, polyclonal antiserum directed against the species divergent carboxyterminal domain of PfTopoII was available and was used at a dilution of 1:1000.

A triplet of proteins of around 160/175 kDa was detected by this antiserum in recombinant cell extracts only. Additional lower molecular weight proteins were also recognised by this antiserum. Two of these proteins cross-reacted with the uninfected Sf9 control, and one with the wild type control. The low molecular weight proteins may be the products of *in vivo* proteolysis or may be the result of proteolysis occurring as the cells are lysed. The latter possibility seems less likely as the cells were lysed using SDS-PAGE loading buffer.

These results indicate that recombinant PfTopoII is being produced in baculovirus, albeit in very small quantities.

5.7 Recombinant PfTopoII can be immunoprecipitated.

1 X 10⁶ insect cells were infected with recombinant baculovirus 1A for periods of 24 and 48 hours or a wild type baculovirus control for 48 hours. A "mock infected" negative control also containing 1 X 10⁶ uninfected insect cells was also set up. The cells were transferred to methionine-deficient medium two hours before harvesting to deplete endogenous pools of the amino acid before pulse labelling. [³⁵S] methionine was added one hour after being transferred to the methionine-deficient medium (2.5.8.1).

Cell lysates were precleared using pre-immune rabbit serum before incubating with immune serum obtained from the same rabbit (directed against the species divergent carboxyterminal domain of PfTopoII) for 3

Figure 8.

Western blot of total protein harvested from recombinant baculovirus infected Sf9 insect cells at 18, 20, 22 and 24 hours post infection (p.i.).

The western blot was probed with a polyclonal antiserum directed against the C-terminal domain of PfTopoII at a dilution of 1:1000. Cross-reactive proteins were detected using anti-rabbit IgG-alkaline phosphatase conjugate at a dilution of 1:7500.

Lane 1. Total protein isolated from uninfected Sf9 cells. Lanes 2, 3, 4 and 5. Total protein isolated from Sf9 cells infected with recombinant baculovirus 1A for 18, 20, 22 and 24 hours. Lane 6. Total protein isolated from wild type (AcNPV) baculovirus infected Sf9 cells at 24 hours p.i.

The position of the protein size standards are indicated. The positions of the triplet of PfTopoII antibody cross-reactive proteins are marked.

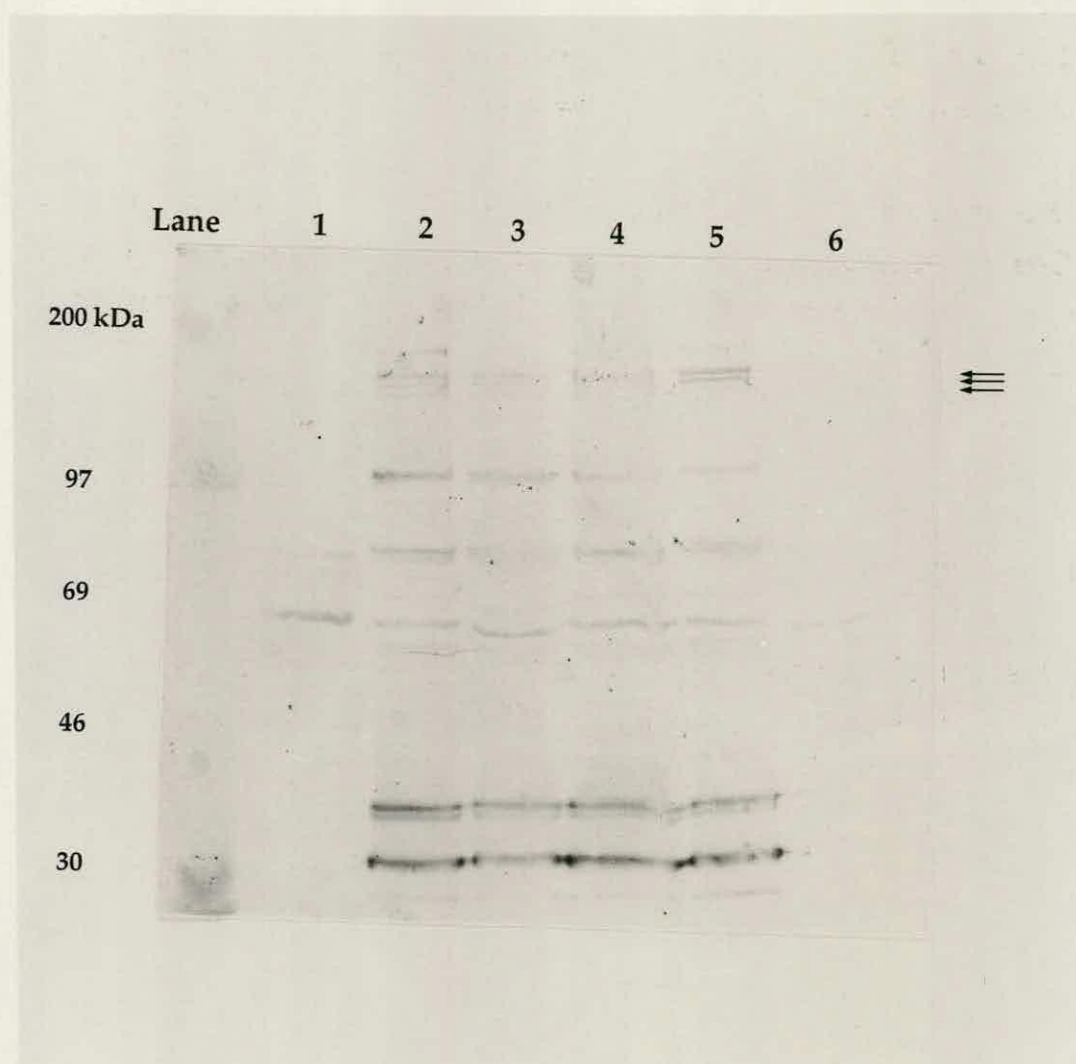


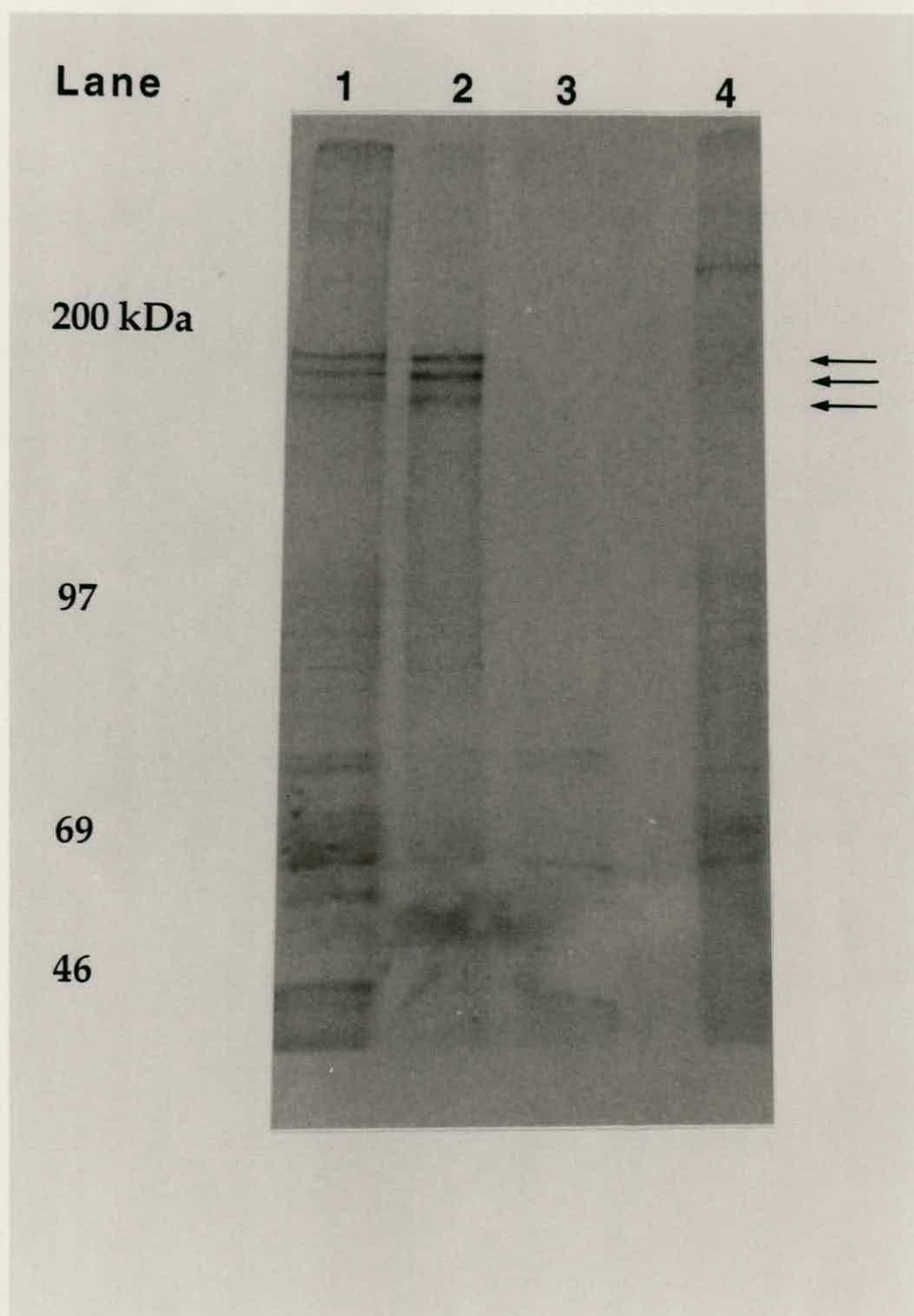
Figure 9.

Immunoprecipitation of recombinant PfTopoII from baculovirus infected Sf9 insect cells metabolically labelled with ^{35}S methionine.

Lanes 1 and 2. Recombinant baculovirus 1A at 24 and 48 hours post infection (p.i.). Lane 3 Wild type baculovirus at 48 hours p.i. Lane 4. Uninfected Sf9 insect cells.

The position of the protein size standards are indicated.

The triplet of proteins derived from recombinant PfTopoII are indicated.



hours to facilitate formation of the antibody-antigen complex. Immune complexes were precipitated using Protein A Sepharose (2.5.8.3) and size fractionated using SDS-PAGE with a 7% gel and the largest size gel plates available (250mm X 200mm), the gel dried and exposed to film.

Figure 9 shows that a triplet of signals in the size range of 150-175 kDa are present in the recombinant PfTOP2 baculovirus sample; these are not present in the wild type control sample, nor are they present in the uninfected insect cell sample. Other lower molecular weight bands which may represent proteolytic fragments of the full length protein are also present.

This result provides further evidence that recombinant PfTopoII is expressed in baculovirus.

5.8 Re-engineering of the PfTOP2 gene to incorporate a 3' affinity tag to facilitate protein purification.

Attempts were not made to purify recombinant PfTopoII due to the very low level of expression observed. Instead, it was decided to clone the gene into the baculovirus transfer vector pAcSG His, which encodes an N-terminal hexahistidine tag which should make it possible to affinity purify the recombinant protein. This should simplify purification of the under-expressed recombinant protein from large scale cultures.

It proved impossible to clone the PfTOP2 into this vector, however, so an alternative strategy was adopted to incorporate a 3' hexahistidine tag into the protein. This involved amplifying the 3' *Bam*HI to TAA fragment of the gene as described previously (figure 2) using the sense oligonucleotide 445Y, but this time using an antisense oligonucleotide encoding 6 histidine residues immediately downstream of a thrombin cleavage site next to the last PfTOP2 codon. This was followed by a stop codon and *Bgl*II and *Sac*I restriction sites. The PCR product was cloned following the same strategy as before (figure 2) into the baculovirus transfer vector pVL1393. Diagram 4.8 shows a plan of the modifications made to the gene to incorporate these features.



Diagram 4.8. Plan of the PfTOP2 gene modified to express a C- terminal hexahistidine tag downstream of a thrombin cleavage site.

5.9 Identification of recombinant PfTOP2-His baculoviruses.

Following successful co-transfection of Sf9 insect cells, clonal selection (2.3.10.2) and viral amplification (2.3.10.3), candidate recombinant baculoviruses were analysed for the presence of the PfTOP2 gene by restriction analysis and Southern blotting (results not shown). Two recombinant baculoviruses (3/1 and 4/1) yielded high titre viral stocks (1×10^9 pfu/ml). Northern blot analysis and immunoprecipitation were not conducted this time. Recombinant baculovirus 4/1-His was found to express the same protein triplet as the untagged baculoviruses 1A and 2A (evaluated by western blotting and probing with anti-PfTopoII serum), and was subsequently used for all affinity purification work.

5.10 Affinity purification of recombinant PfTopoII-His.

5.10.1. Imidazole step purification of recombinant protein.

Metal chelate affinity chromatography can be used as a means to purify hexahistidine tagged proteins. An immobilised gel matrix is charged with nickel ions to form a chelate. Proteins containing surface polyhistidine residues have a high affinity for the metal chelate and will bind strongly to the matrix. The adsorbed proteins can be removed by competitive elution using imidazole, or by lowering the pH of the elution buffer.

Whole cells extracts, prepared from 250 ml of Sf9 cells (2.5×10^8 cells)

infected with recombinant baculovirus 4/1-His for a period of 48 hours were used for affinity purification (2.5.6) under non-denaturing conditions using a nickel column. In this case recombinant proteins were eluted in one step using 500mM imidazole.

Figure 10 (a) shows the Coomassie blue stained gel following SDS-PAGE fractionation of the samples and also an identical SDS polyacrylamide gel following western blotting and probing with the C-terminal PfTopoII antiserum at a dilution of 1:1000 [figure 10 (b)]. 1 ml fractions were collected from the imidazole step elution and 40 μ l samples of each were loaded onto each gel. Figure 10 (b), lane 2 shows that some proteins did not bind to the column and eluted in the flow through. Three distinct protein bands which co-migrate just below the 175 kDa size standard, along with some lower molecular weight bands can be observed. It is possible that these proteins did not bind to the column because the C-terminal hexahistidine tag may have been conformationally hidden or degraded.

Fractions 2 and 3 (Figure 10 b, lanes 5 and 6) from the imidazole eluant reveal two bands of approximately the size expected for recombinant PfTopoII (175/160 kDa). Several other bands of lower molecular weight also elute in fractions 2 and 3, the most striking of which are about 30 kDa in size. These proteins are most likely to be the products of N-terminal proteolysis of the recombinant protein because they are detected by the PfTopoII C-terminal antibody.

This purification procedure was repeated at least twice and similar results were obtained each time (data not shown). In addition, fractions 2 and 3 (lanes 5 and 6) were tested for evidence of recombinant PfTopoII enzyme activity using standard decatenation assay tests. Following dialysis against TopoII assay buffer to remove excess imidazole and salt, fraction 2 (lane 5) was found to contain a low level of activity (results not shown).

5.10.2 Gradient purification of recombinant protein.

An imidazole gradient elution purification was used in an attempt to obtain full length recombinant PfTopoII that is free of the hexahistidine tagged contaminating proteins of lower mobility (2.5.6).

Figure 10.

Affinity purification of recombinant PfTopoII-His.

Whole cell extracts from Sf9 insect cells infected with recombinant PfTOP2 baculovirus 4/1-His were loaded onto a nickel chelating column (Pharmacia) under non-denaturing conditions. Hexahistidine-tagged proteins were eluted in one 500mM imidazole step.

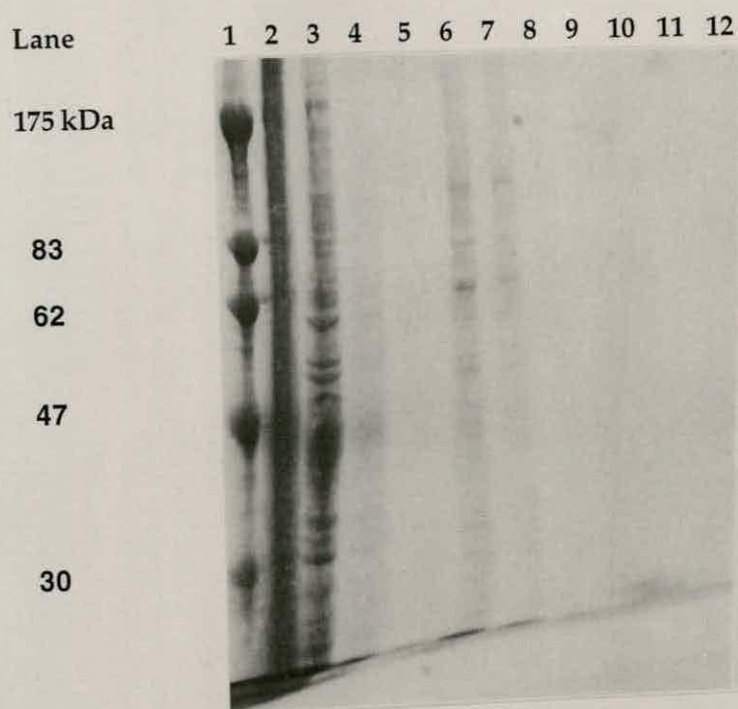
Panel a. Coomassie blue stained SDS-polyacrylamide gel of affinity purified recombinant PfTopoII-His.

Lane 1. Protein size standards. Lane 2. Unbound proteins. Lane 3. Column wash fraction 1. Lane 4. Column wash fraction 2. Lanes 5-12. 500mM imidazole eluted proteins (fractions 1-7).

Panel b. Western blot of affinity purification shown in Panel a probed with anti-PfTopoII C- terminal serum.

Lane 1. Protein size standards. Lane 2. Unbound proteins. Lane 3. Column wash fraction. Lanes 4-12. 500mM imidazole eluted proteins (fractions 1-7).

a)



b)



163

In this case, 1 litre (1×10^9) of infected cells Sf9 were used to prepare cell extracts. Proteins eluted from a gradient of 0-500mM imidazole were size fractionated using SDS-PAGE. One SDS-polyacrylamide gel was stained with coomassie blue and an identical gel was western blotted and probed with the same antisera used in section 5.10.1 at a dilution of 1:1000.

Figure 11 shows the result of this experiment. Coomassie blue staining revealed the presence of some protein bands in fractions 2 and 3 and 4 from the imidazole gradient (figure 11 a, lanes 5, 6 and 7). Western blotting shows that several proteins appear not to bind to the nickel column and elute in the flow through fraction (figure 11 b, lane 2). The pattern of these proteins is very similar to that obtained before (see figure 10). The imidazole gradient elutes numerous PfTopoII C-terminal antibody cross-reacting proteins in lanes 5 and 6. This suggests that these proteins do not require a high concentration of imidazole to displace them from the nickel column. A doublet of proteins of approximately 160 kDa are detected in fraction 2 of the imidazole gradient (Figure 11 b, lane 5). Gradient purification was tried again several times to try to remove the lower molecular weight hexahistidine tagged proteins from full length recombinant PfTopoII but this was not possible.

5.11 Analysis of enzyme activity.

In order to establish whether the very small quantity of full length recombinant protein contained TopoII activity, fractions 1-6 (figure 11, lanes 4-9) from the imidazole gradient were tested (after removal of imidazole and salt by dialysis against TopoII assay buffer).

200ng of KDNA was used for each decatenation assay, together with 1 μ l and 10 μ l of each protein sample (2.9.3). As can be seen in Figure 12 (a), a low level of activity can be detected in almost all of the reactions containing 10 μ l of protein from each fraction.

This suggest that a co-purifying insect cell or viral encoded type II topoisomerase activity may be contaminating some or all of the fractions. In order to test for this possibility, a parallel experiment was conducted using an imidazole gradient to "mock" purify a non-histidine tagged recombinant baculovirus expressing *P. falciparum* DNA polymerase δ

Figure 11.

Affinity purification of recombinant PfTopoII-His using an imidazole gradient.

Whole cell extracts from Sf9 insect cells infected with recombinant PfTOP2 baculovirus 4/1-His were loaded onto a nickel chelating column (Pharmacia) under non-denaturing conditions. Hexahistidine tagged proteins were eluted using a gradient of 0-500mM imidazole.

Panel a. Coomassie blue stained SDS-polyacrylamide gel of affinity purification.

Lane 1. Protein size standards. Lane 2. Unbound proteins. Lane 3. Column wash fraction 1. Lanes 4-9. 0-500mM imidazole gradient (fractions 1-6).

Panel b. Western blot of affinity purification shown in Panel a probed with anti-PfTopoII C-terminal serum.

Lane 1. Protein size standards. Lane 2. Unbound proteins. Lane 3. Column wash fraction. Lanes 4-9. 0-500mM imidazole eluted proteins (fractions 1-6).

a)

Lanes

1 2 3 4 5 6 7 8 9 10

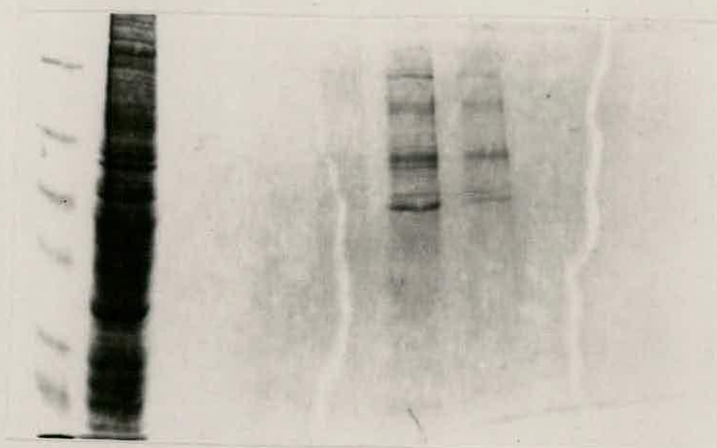
175 kDa

83

62

47

30



b)

Lanes

1 2 3 4 5 6 7 8 9

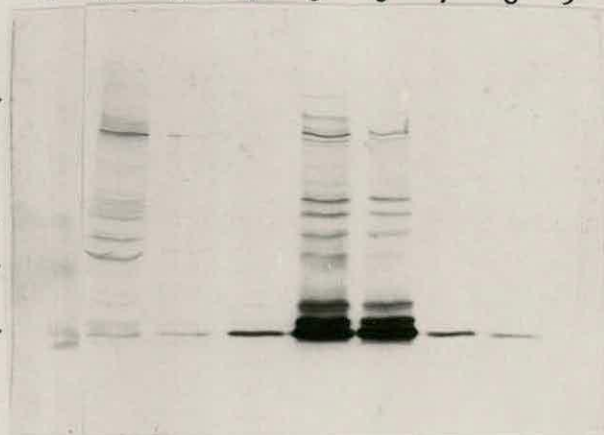
175 kDa

83

62

47

30



(PfPol δ). Identical fractions were collected from the imidazole gradient, thus allowing a reasonably accurate comparison to be made between the hisidine-tagged PfTopoII fractions and the recombinant PfPol δ fractions. Figure 12 (b) shows the results of a decatenation assay conducted using "mock purified" recombinant PfPol δ . The same amount of KDNA and protein were used as before, after imidazole and salt removal. As can be seen from a comparison of figure 12 (a) and (b), a similar profile of activity is apparent in each experiment. This strongly suggests that most, if not all, of the activity seen in recombinant PfTopoII preparations probably originates from the insect cell.

Figure 12.

Panel a. Decatenation assay using recombinant PfTopoII-His

Decatenation assay using 200ng of kinetoplast DNA (KDNA) and protein from fractions 1-6 of an imidazole gradient purification of recombinant PfTopoII-His. 1 and 10 μ l of protein were drop dialysed to remove imidazole and salt contamination prior to conducting the assays.

Lane 1. Assay conducted without protein. Lanes 2 and 3. Assays conducted on protein from fraction 1 of the gradient. Lanes 4 and 5. Assays conducted on protein from fraction 2 of the gradient. Lanes 6 and 7. Assays conducted on protein from fraction 3 of the gradient. Lanes 8 and 9. Assays conducted on protein from fraction 4 of the gradient. Lanes 10 and 11. Assays conducted on protein from fraction 5 of the gradient. Lanes 12 and 13. Assays conducted on protein from fraction 6 of the gradient. Even numbered lanes contain assays conducted with 1 μ l of protein and odd numbered lanes contain assays conducted with 10 μ l of protein. The positions of the open circular (OC) and covalently closed circular (CCC) DNA species are indicated.

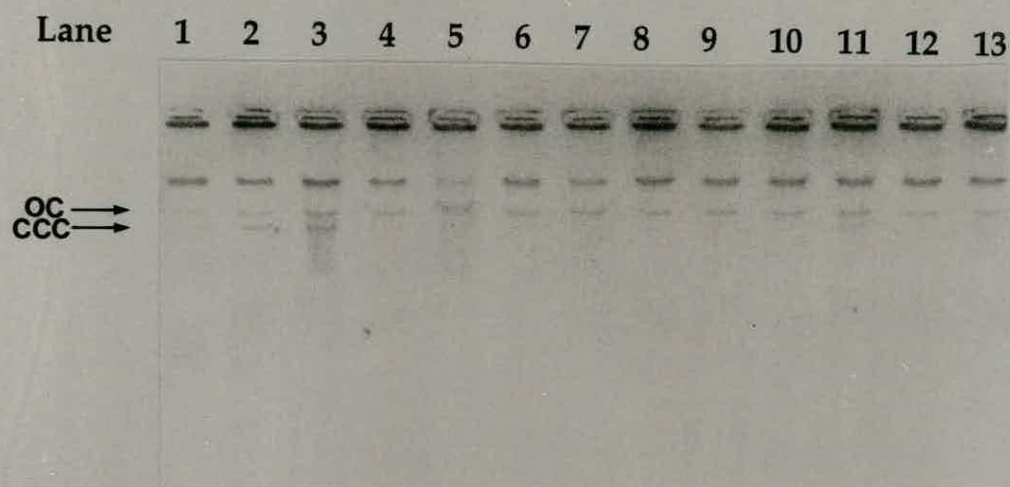
Panel b. Decatenation assay using recombinant PfPol δ without a His tag.

Decatenation assay using 200ng of kinetoplast DNA (KDNA) and protein from fractions 1-6 of an imidazole gradient purification of recombinant PfPol δ . 1 and 10 μ l of protein were drop dialysed to remove imidazole and salt contamination prior to conducting the assays.

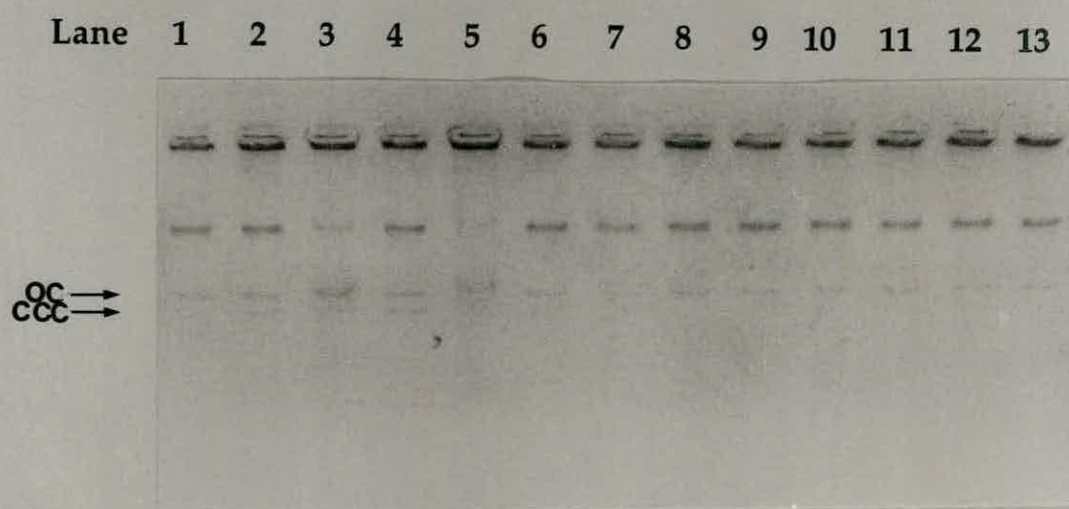
The legend for this experiment is the same as shown in Panel a above.

The positions of the open circular (OC) and covalently closed circular (CCC) DNA species are indicated.

a)



b)



5.12 Discussion.

Heterologous expression of PftopoII in baculovirus-infected Sf9 insect cell lines has been undertaken in an attempt to purify enough of the recombinant enzyme to facilitate biochemical analysis.

Coomassie-stained SDS-polyacrylamide gels using whole cell lysates from insect cells infected with recombinant PftOP2 baculoviruses did not reveal any novel proteins which were within the expected size range for recombinant PftopoII. This indicated that (i) the protein was not being expressed, or (ii) the protein was expressed in very small quantities, or (iii) the protein was undergoing rapid *in vivo* proteolysis.

In order to investigate whether transcription of the PftOP2 gene was occurring, total RNA was isolated and analysed by northern blotting. This technique revealed that a prominent transcript of around 5 kb was being made; a size that is consistent with the size of the PftOP2 ORF of 4191 bp (if allowances are made for inclusion of a 5' leader and polyA tail). The 5 kb transcript was identified by probing the same northern blot with the 5' and 3' ends of the PftOP2 gene. These probes did not hybridise to mRNA derived from the wild type parent virus, confirming that the transcripts originate from the PftOP2 gene. The presence of multiple abundant polymorphic transcripts derived from the recombinant PftOP2 mRNA however, complicates the picture, but there may be an explanation for this.

Late and very late baculoviral promoters (for example polh) have essential TAAG sequences from which mRNAs initiate. These TAAG sequences occur very infrequently in the AcNPV genome but some mapping of the sites has been done. For example, Garrity *et al.*, (1997) have found five copies of the sequence in the upstream promoter region of the AcMNPV gp64 envelope fusion protein gene, although transcription is only detected from two of them. Vanstrien *et al.*, (1996) have analysed the ubiquitin gene-cluster of the *Spodoptera exigua* nucleopolyhedrovirus (SeMNPV) and found that three major small transcripts were made late in the infection (400, 550 and 690 nt). Primer extension analysis showed that transcription started from two consensus late promoter elements (TAAG). Exactly what effect TAAG sequences

have on expression of foreign genes is difficult to predict, but their presence in either direction in the gene can reduce expression levels. For example, TAAG sequences in the sense direction may, depending upon their context, (which is presently unknown) cause transcription initiation within the gene. If on the other hand, CTTA sequences are present in the antisense direction, then formation of antisense transcripts might also occur. These could disrupt expression by causing the formation of a sense/antisense duplex transcript, thus leading to translational blocks (O'Reilly, 1992).

The PfTOP2 gene has at least 19 TAAG motifs in the sense direction within the ORF. If any of these motifs are present in the appropriate context, then it is feasible that fortuitous transcription initiation could occur. It is also possible that PfTOP2 antisense transcripts may have been produced, but this possibility was not investigated.

An alternative explanation is that RNA degradation is responsible for the presence of the highly abundant transcripts of less than 4 kb in size. Although we cannot completely eliminate this possibility, it seems unlikely for three reasons. Firstly the presence of the 5 kb transcript suggests that mRNA in this size range has not been degraded. Secondly, UV analysis indicates the nucleic acid content appeared to be of high yield with no protein or salt contamination. Finally, discrete ribosomal RNA bands were present in the ethidium-stained gel following size fractionation. It is more difficult to understand why the 28S and 18S ribosomal bands are not present in equal amounts. At present it is not known whether baculovirus modifies the insect ribosomes during the course of its infection.

Once the antiserum against the C-terminal portion of PfTopoII had become available it soon became apparent that recombinant PfTopoII was being produced as demonstrated by western blotting, and immunoprecipitation experiments. Both techniques showed that a characteristic triplet of proteins was produced of around 160/175 kDa, together with a somewhat larger protein. Similar protein triplets have been observed on western blots from parasites synchronised in ring, trophozoite and schizonts (chapter 4) using the same antiserum.

The fact that recombinant PfTopoII could not be detected by conventional protein staining techniques suggested that it was being made in very small quantities. Because of this, we decided to incorporate a hexahistidine affinity tag into the carboxy-terminus of PfTopoII to simplify protein purification and maximise yields. Nickel chelate chromatography purified a very small amount of full length PfTopoII. Fractions containing the recombinant protein were tested for their ability to decatenate kinetoplast DNA, which is a definitive test for a type II topoisomerase activity. Although enzyme activity was observed in some of the fractions recovered from the purification, it became clear that this was likely to be the result of a co-purifying insect cell activity after a parallel purification using a recombinant baculovirus expressing a different protein yielded similar results.

It remains unclear why recombinant PfTopoII was produced in such a disappointingly low quantity but one possible explanation could be related to the TAAG motifs present in the gene, or another possibility is that the protein was toxic to the cell. PfPCNA, as mentioned previously, is a relatively small protein of some 30 kDa that has been successfully overexpressed in our laboratory using baculovirus. Analysis of the open reading frame in this case suggests that only two TAAG sequences are present in the sense direction. For small *P.falciparum* genes such as PfPCNA TAAG motifs may not occur frequently enough to cause fortuitous transcription initiation events.

Chapter 6

Heterologous expression of *P. falciparum* topoisomerase II in *S. cerevisiae*.

6.1 Introduction.

The yeast *S. cerevisiae* is a very attractive system for the expression of foreign genes. Its genetics are more advanced than any other eukaryote with numerous temperature-sensitive mutants available that allow introduced sequences to be tested for their ability to complement the genetic defect. Other desirable features include its ease and safety of handling, together with the ability to grow rapidly on inexpensive medium. Most of the expression vectors in use today are based on the 2 μ plasmid, and contain sequences that allow propagation in yeast and *E. coli*, as well as yeast promoter and terminator sequences. Although other yeasts are now routinely used for high level expression of foreign DNA sequences, (eg. the methyltroph *Pichia pastoris*), discussion of yeast in this chapter will be restricted to *S. cerevisiae*.

Human TopoII α has been overexpressed in *S. cerevisiae* (Wasserman *et al.*, 1993) and the 170 kDa recombinant enzyme is reported to be functionally indistinguishable from the authentic protein purified from HeLa cells. In addition, both α and β isoforms were able to complement yeast temperature sensitive *top2* mutants which lack a functional topoisomerase II at the restrictive temperature (Wasserman *et al.*, 1993 and Meczes *et al.*, 1997). Drug permeable, yeast *top2* mutants were used to test known TopoII inhibitors against plasmid borne GAL1 inducible, TopoII α , TopoII β or yeast TopoII, thus providing a rapid method to analyse the differential drug sensitivity of each homologue (Meczes *et al.*, 1997).

In view of the limited success we have experienced to date with heterologous expression of PfTopoII, we considered the possibility of adapting the yeast system used above as a feasible alternative to facilitate rapid screening of candidate PfTopoII inhibitors *in vivo*. We were initially concerned that there are few reported cases of successful overexpression of *P. falciparum* genes in *S. cerevisiae*, however, there are

a few examples where *P. falciparum* sequences have been used successfully to complement *S. cerevisiae* temperature sensitive defects. For example, pyrimethamine and cycloguanil are compounds which have been used extensively to treat malaria, but rapid selection of populations of parasites which have developed resistance have compromised their effectiveness. Wooden *et al.*, (1997) have used yeast temperature-sensitive mutants that lack endogenous DHFR activity to investigate the frequency with which point mutations in the *P. falciparum* dihydrofolate reductase enzyme (PfDHFR) occur following exposure to the competitive inhibitors, pyrimethamine and cycloguanil. In this system, expression of the recombinant PfDHFR domain was controlled by the yeast *dhfr* 5' and 3' regulatory regions, and the recombinant enzyme complemented the yeast defect. Yeast expressing recombinant PfDHFR are found to be sensitive to both of the drugs, at concentrations within the range that inhibit drug sensitive parasites *in vivo*.

In a different study, yeast was used to develop a system for functional analysis of the *pfmdr1* gene and its product, the Pgh1 protein, and to clarify its role as a transport molecule in drug resistance (Volkam *et al.*, 1995). Overexpression of the *pfmdr1* gene has been associated with the mefloquine-resistance and halofantrine-resistance phenotypes in *P. falciparum*. The *pfmdr1* gene expressed in the yeast system was able to restore fertility to a sterile (*ste6*) mutant which lacked a functional exporter molecule for the α -factor pheromone.

Both of these studies suggest that it is possible to express *P. falciparum* genes which are able to then rescue genetic defects in temperature-sensitive yeast mutants. Encouraged by the success of these studies, we attempted heterologous expression of PfTopoII in *S. cerevisiae* strains engineered for overexpression, and in temperature-sensitive strains lacking a functional yeast TopoII at restrictive temperatures.

6.2 Construction of the PfTOP2 gene for expression in *S. cerevisiae*.

To express the *P. falciparum* TOP2 gene in yeast, the first four PfTOP2 codons were replaced with the first five yeast TOP2 codons. This approach was based on earlier work of Wasserman *et al.*, (1993) on the expression of human TopoII α in yeast. They demonstrated that by fusing the 29th codon of the human TOP2 α gene, to the 5th codon of the yeast TOP2 gene, expression was improved. This improvement is probably related to the optimisation of the nucleotide sequences around the ATG codon.

Several cloning steps were required to engineer the PfTOP2 gene for expression, including use of PCR to change the first four *P. falciparum* codons into the first five yeast TopoII codons. Figure 1 (a, b and c) outline the strategy that was used. The entire PfTOP2 cassette was excised as a *SalI/XhoI* fragment (figure 1c) for subcloning into the yeast expression vector YEpWob6 (Figure 2, Wasserman *et al.*, 1993). Numerous attempts to clone the coding sequence into YEpWob6 proved unsuccessful. The possibility that the gene is unstable in this particular vector led us to try to attempt to clone it into alternative vectors YC*plac*33 and YE*plac*195 [Figure 3 (Geitz and Sugino, 1988)], but this approach also failed.

At this stage we had no idea why the gene appeared to be unstable in all three of the vectors. We attempted to investigate this by ligating a *HindIII* cassette containing a URA3 gene and 2 μ origin of replication (Gibbs *et al.*, 1993) into the vector pUBS1 (pUBS/URA3). The GAL1/PfTOP2 *SalI/XhoI* fragment from plasmid 218/PfTOP2 was subcloned into the *SalI/XhoI* site in pUBS/URA3. This procedure was conducted in pUBS because of the lack of suitable restriction sites in plasmid 218/PfTOP2. Very few transformants were recovered from these ligations, but in every case where the GAL1/PfTOP2 insert was present, it was found that the URA3 gene had been excised *in vivo*. This suggests that the PfTOP2 gene cannot co-exist on the same plasmid as the URA3 gene.

Alternative selectable markers are available, and in an attempt to overcome this problem, the *SalI/XhoI* fragment containing the PfTOP2 gene (218/PfTOP2, shown in figure 1 c) was subcloned into the *SalI* site of YE*plac*181 which uses LEU2 as a selective marker (Geitz and Sugino, 1988).

1) $KpnI$ $BglII$ 5 yeast codons

5'GGGGTACCAGATCTGCTAACCATGTCAACTGAACCG
aaaaactattgaggagtatc 3'

PfTOP2 codons

Oligonucleotide H4535

2) H4535
ATG PstI

0 899 6165

Wild type PfTOP2 sequence

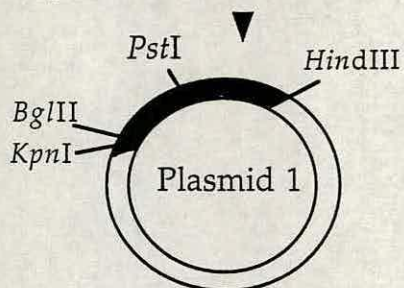
2) Sequence of the wild type PfTOP2 sequence that was used as a template for oligonucleotides H4535 and G6165.

The pGAL1/PfTOP2 fragment (*SalI*/*XhoI*) in 218/PfTOP2 was excised ready to clone into the yeast expression vector YEpWOB6.

a) Construction of the 5' end of the PfTOP2 gene



(i) Restrict the 5' PCR fragment with *KpnI*/*PstI* and clone into Plasmid 1 which already contains the *PstI*/*HindIII* fragment of the PfTOP2 gene

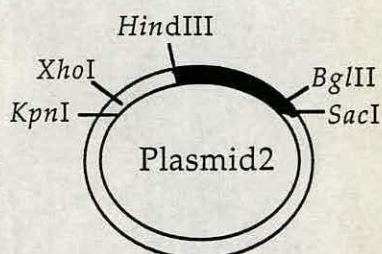


b) Construction of the 3' end of the gene

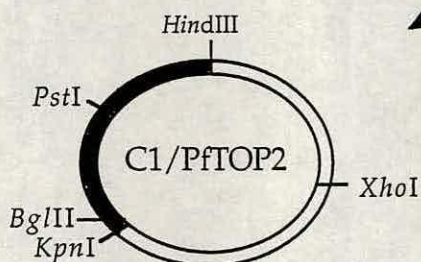
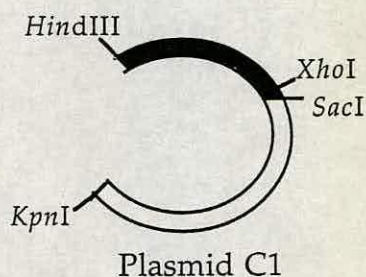
Plasmid 2 contains the 3' *HindIII* to TAA/*SacI* region of the PfTOP2 gene.

(ii) Restrict with *BglII* and linker with *XhoI*

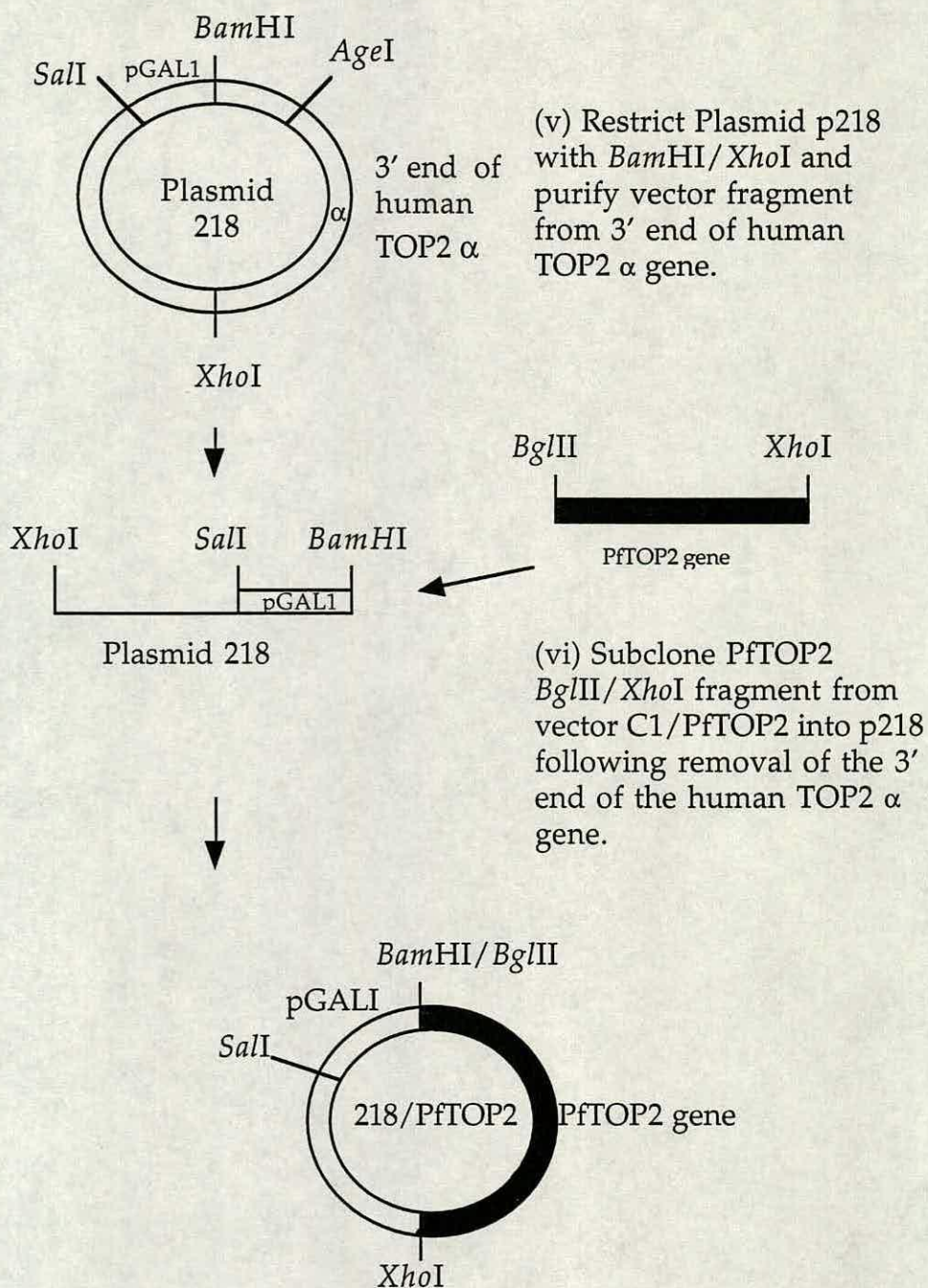
(iii) Restrict with *KpnI*/*HindIII*



(iv) Restrict Plasmid 1 with *KpnI*/*HindIII*, isolate the PfTOP2 fragment and clone into Plasmid C1.



c) Fusion of the PfTOP2 gene to the GAL1 promoter in plasmid 218.



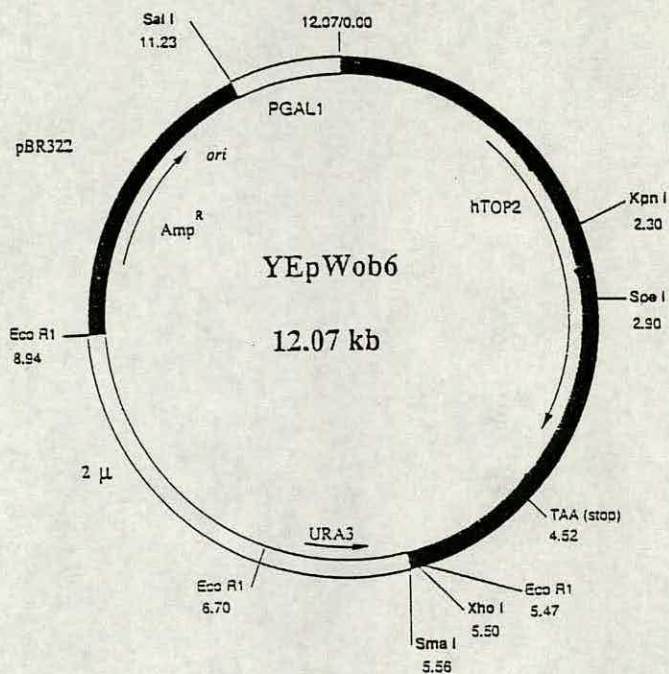


Figure 2.

Physical map of the yeast/*E.coli* shuttle vector YEpWob6 that was used to express human TopoII α . In order to use this plasmid to express the PfTOP2 gene, the *Sal*I/*Xho*I fragment containing the human TopoII α gene and GAL1 promoter was removed.

Attempts to ligate the *Sal*I/*Xho*I fragment containing the PfTOP2 gene and GAL1 promoter (shown in figure 1 c) into this vector proved unsuccessful.

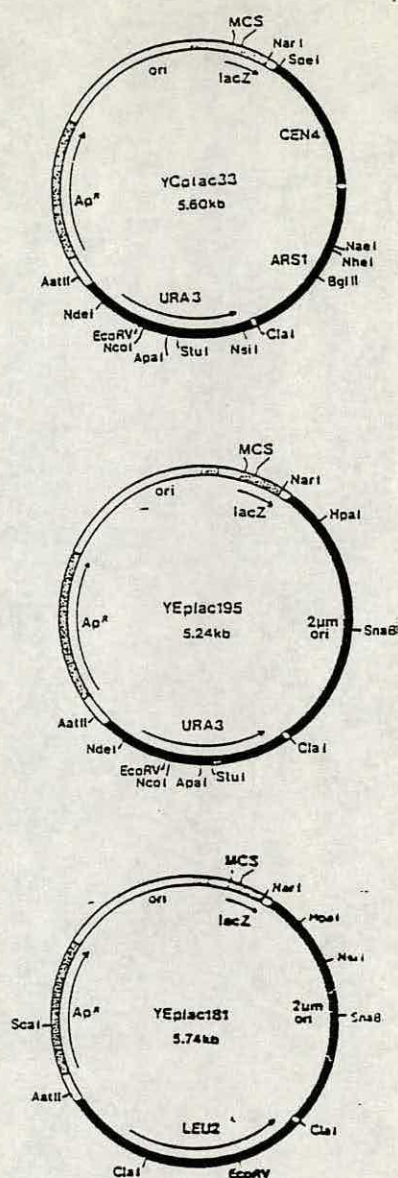


Figure 3.

Physical maps of the yeast/*E.coli* shuttle vectors (Geitz and Sugino 1988) used for cloning of the PFTOP2 gene.

The multiple cloning site (MCS) is based on pUC19 (Yanisch-Perron *et al.*, 1985) and contains the following restriction sites; *Hind*III, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sst*I, and *Eco*RI. Each plasmid has a yeast 2μ origin of replication. YCplac33 is a centromeric plasmid containing a URA3 auxotrophic marker, YEplac195 and YEplac181 are episomal plasmids containing URA3 and LEU2 auxotrophic markers.

Sall and *XhoI* produce compatible sticky ends thus facilitating the cloning of the fragment into this vector which lacks an *XhoI* site. This approach was successful and the new construct was used for expression after sequencing of all the cloning borders and PCR amplified regions of the construct.

6.3 Monitoring expression of the PfTOP2 gene. from a GAL1 promoter.

6.3.1 Expression of PfTOP2 in JEL1.

The recombinant YE*plac181*/PfTOP2 expression plasmid was transformed into the protease-deficient yeast strain JEL1 (gift of Dr. Caroline Austin) for overexpression. The parental plasmid YE*plac181*, and YE*pWob6* containing the human TopoII α gene were also transformed into the same yeast strain to act as controls (2.3.6.4).

Recombinant colonies were grown for 24 hours in yeast synthetic minimal medium with the appropriate amino acid supplements, including 2% glucose, 2% glycerol and 2% lactate as carbon sources. 5ml of the culture was used to inoculate 9.5ml of the same medium, but this time glucose was omitted to allow endogenous pools to be depleted before induction. After a further 24 hours when the OD₆₀₀ reached 0.59, the culture was induced with 2% galactose for a period of 4.5 hours (2.5.4.4). Initially, the choice of an appropriate OD₆₀₀ was based on the fact that the human TopoII α gene was only inducible in the range of 0.4-0.98. During the course of this work, a range of OD₆₀₀ between 0.2 and 1.0 were also tested. Yeast cultures were harvested and lysed in the presence of protease inhibitors and the lysates analysed by SDS-PAGE and Coomassie blue staining of the polyacrylamide gels.

Figure 4 (a) shows the result of a typical experiment. A protein with apparent mobility of 170 kDa which is characteristic of expression of recombinant human TopoII α can be seen in lane 5. The expected size of recombinant PfTopoII is around 160 kDa, but no evidence of expression of a protein of this size was observed (figure 4, lane 3). A duplicate polyacrylamide gel was western blotted and probed with the PfTopoII C-terminal antiserum. Figure 4 (b) shows that although there appears to be a band of approximately the right size for recombinant PfTopoII (~160 kDa),

Figure 4. (a).

Total protein isolated from *S. cerevisiae* cultures was size fractionated using SDS-PAGE. The polyacrylamide gel was stained with Coomassie blue to visualise the protein bands.

Lane 1 contains protein derived from a culture containing the parent vector YEplac181 without the PfTOP2 gene. Lane 2 contains protein derived from a non-induced culture containing the GAL1/PfTOP2 construct in YEplac181 (Yeplac181/PfTOP2). Lane 3 contains protein derived from induced cultures containing the Yeplac181/PfTOP2 construct. Lane 4 contains protein derived from non-induced cultures containing the human TopoII α gene in YEpWOB6. Lane 5 contains protein derived from induced cultures containing the human TopoII α gene in YEpWOB6.

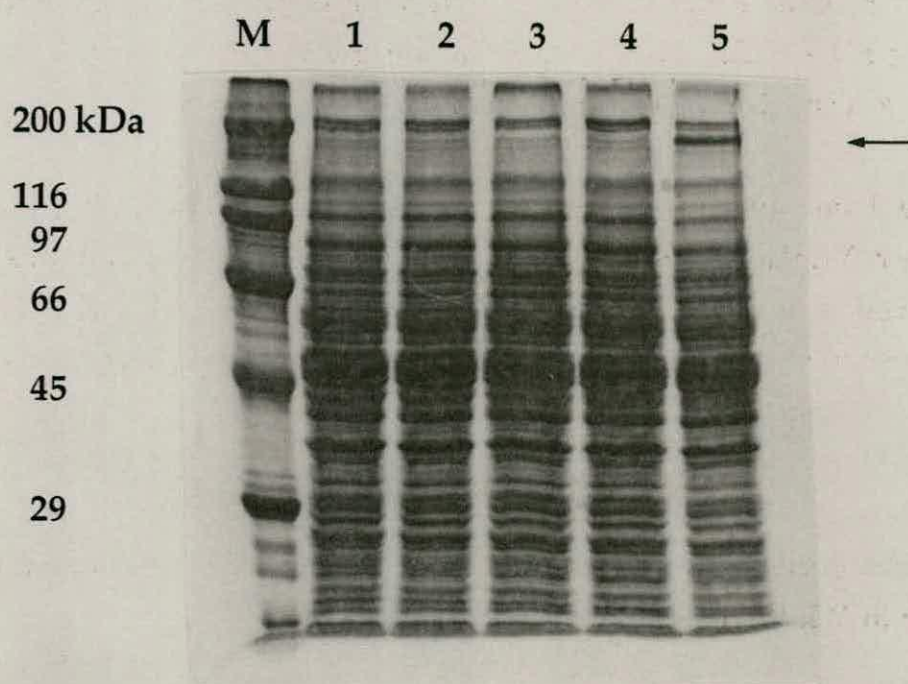
The positions of the protein size standards are shown on the left of the figure (in kilodaltons).

Figure 4 (b).

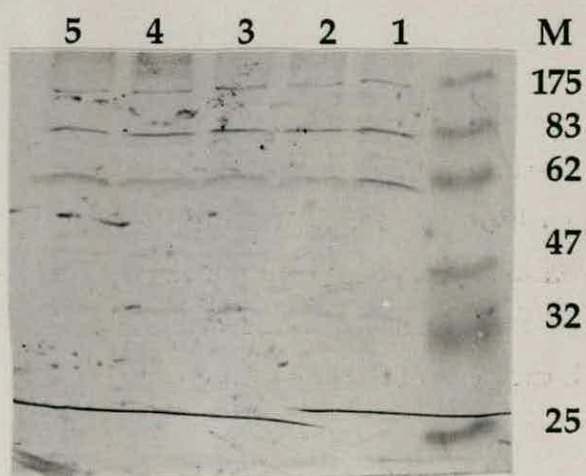
Figure 4 (b) shows a duplicate gel of the one described in figure 4 (a). The gel was western blotted to a nitrocellulose membrane and probed with antiserum raised against the C-terminal region of PfTopoII.

The contents of the lanes are as described in figure 4 (a). Protein size standards are indicated on the left of the figure.

(a)



(b)



the band is present in all of the samples, as are two other smaller bands of around 80 and 60 kDa. In order to ensure that the apparent lack of expression of plasmid YEplac181/PfTOP2 could not be attributed to loss of the plasmid from the cells (JEL1), yeast cultures were lysed, the recombinant plasmid recovered, and transformed into DH5 α (2.3.1.3). The recombinant plasmid was isolated on each occasion that this procedure was performed.

6.3.2 Expression of PfTOP2 in temperature-sensitive yeast strains.

The recombinant YEplac181/PfTOP2 plasmid and YEplac181 parental plasmid and YEpWob6 were transformed into four temperature sensitive *S. cerevisiae* strains JCW 26 (*top* 2-4), SD117 (*top* 2-1), JCW28 (*top*1, *top*2-4) and SD119 (*top*1-1, *top*2-1). JCW 26/SD117 strains have inactive topoisomerase II, and JCW 28/SD119 have inactive topoisomerases I and II enzymes at restrictive temperatures (34°C).

Complementation tests were conducted as such; cultures containing either the recombinant plasmid or parent plasmid, were grown overnight in minimal medium (with appropriate amino acid supplements) and 2% glycerol/2% lactate. Cell numbers were counted before washing in minimal medium without a carbon source. 3×10^5 cells were spotted onto selective medium containing 2% glucose, or 2% glycerol/2% lactate supplemented with 2% galactose. 3×10^5 cells were also spotted onto duplicate plates for each strain, and were incubated at the permissive temperature (23°C) to ensure that the cells could grow normally, while the duplicate plate, was incubated at the restrictive temperature (34°C). Plates were incubated for up to ten days (2.5.9).

All of the strains grew normally at permissive temperatures. Strains grown at restrictive temperatures with YEplac181/PfTOP2 or YEplac181 plasmids showed no signs of growth ten days after plating. Strains grown at the restrictive temperature with YEpWob6 containing the human TopoII α gene were viable, however. In order to test the possibility that recombinant PfTopoII may have been expressed at a high enough level to be toxic to the cells, we decided to test a range of galactose concentrations in case the level of inducer being used was too high. Complementation

tests were set up as above, except that cells were spotted at 1×10^5 and the glycerol/lactate plates were in this case supplemented with galactose at final concentrations of 0.05%, 0.5%, 1% and 2% per plate.

Duplicate plates were set up so that individual replicates could be incubated at permissive or restrictive temperatures. After ten days, cells grown at permissive temperatures showed normal signs of growth, but cells grown at restrictive temperatures showed no growth. We conclude either that (i) recombinant PfTopoII has not been functionally expressed in these t.s. strains, or (ii) the recombinant enzyme was expressed but was unable to replace the function of the defective yeast TopoII. The fact that expression of recombinant PfTopoII in the expression strain JEL1 was not observed, may indicate that the former of these two possibilities is the more likely.

In an attempt to ascertain why expression of the recombinant protein was not observed, we decided to investigate whether the GAL1 promoter was actively transcribing the gene upon induction with galactose.

6.4. Truncated PfTOP2 transcripts are expressed from the GAL1 promoter in yeast.

Total RNA was prepared (2.4.2) from 200ml cultures of JEL1 containing YE*plac181*/PfTOP2 or before and after galactose induction (5 hours). In addition, JEL1 harbouring the parent plasmid YE*plac181* alone was used with induction. 18 μ g of each sample of total RNA was size fractionated on a 1% agarose gel containing formaldehyde, and northern blotted to a Genescreen Plus membrane (DuPont). This was probed with a 5' 1.8 kb (1-1795 nt) fragment of the PfTOP2 gene. Figure 5 (a) shows the ethidium bromide-stained formaldehyde gel prior to northern blotting. Figure 5 (b) shows a northern blot of the same gel after probing with the PfTOP2 gene specific probe. Lane 2 of figure 5 (b) shows a transcript of approximately 350 nucleotides which hybridises to this probe. The transcript is only present in RNA derived from an induced culture containing the PfTOP2 recombinant plasmid. Lanes 3 and 4, which contain RNA derived from an uninduced culture containing YE*plac181*/PfTOP2, and a culture containing the parental plasmid alone, do not hybridise to this probe.

Figure 5.

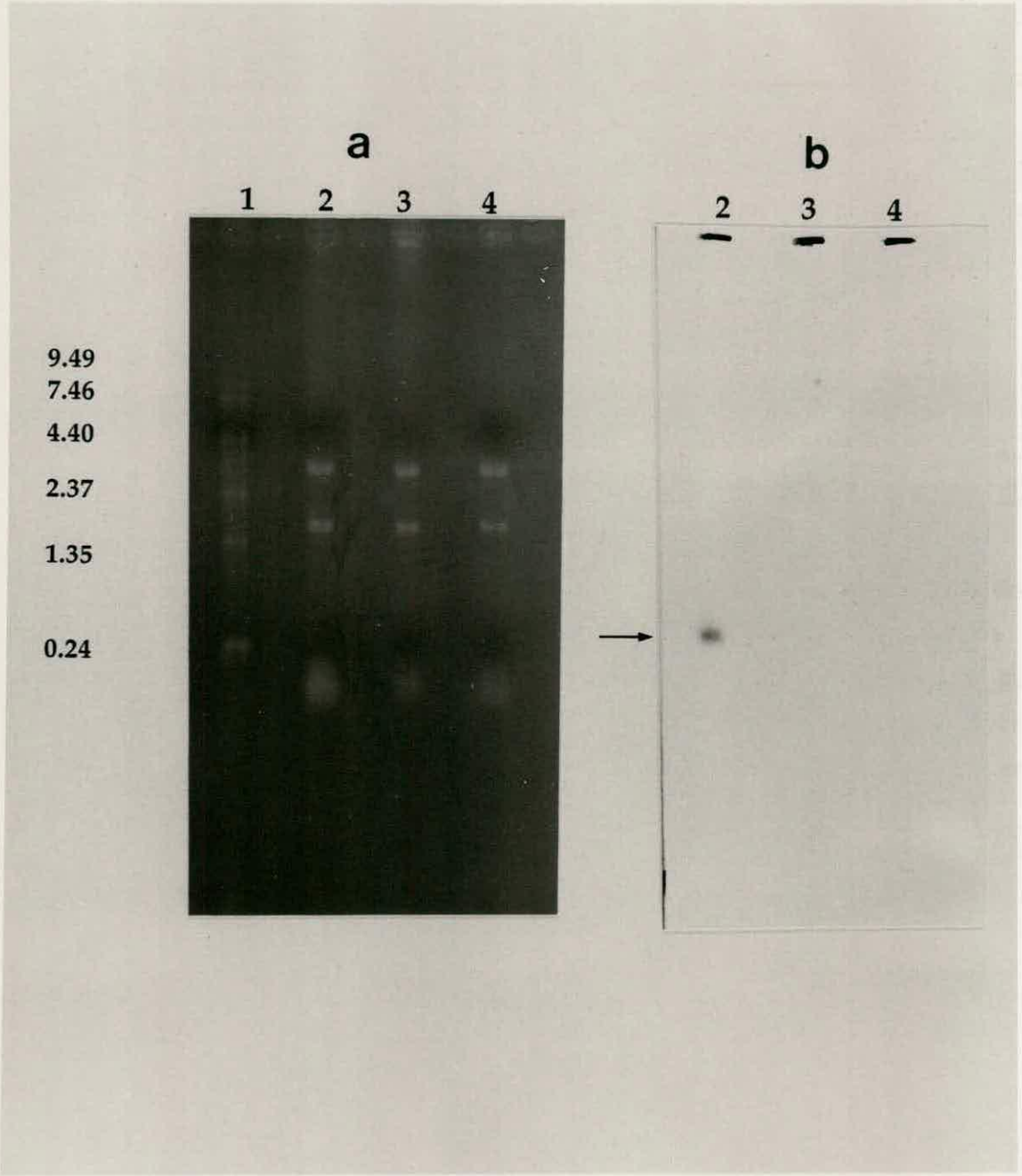
Northern blot analysis of total RNA derived from yeast cultures.

Figure 5 (a) shows the ethidium bromide-stained formaldehyde-treated agarose gel prior to northern blotting.

Lane 1 shows the 0.24-9.5 kb RNA ladder (Gibco BRL). Lane 2 shows total RNA from a galactose-induced culture containing the GAL1/PfTOP2 construct in the shuttle vector YE*plac*181 (YE*plac*181/PfTOP2). Lane 3 shows total RNA from a non-induced culture containing the YE*plac*181/PfTOP2 construct. Lane 4 shows total RNA from a galactose-induced culture containing the parent vector YE*plac*181 without the GAL1/PfTOP2 construct.

Figure 5 (b) shows the same formaldehyde gel blotted to Genescreen Plus membrane, and probed with a 5' 1.8 kb fragment of the PfTOP2 gene. The signal derived from the 350 nt transcript is arrowed.

The RNA size standards were removed from the membrane prior to probing.



In order to map the 3' end(s) of the transcript(s) we decided to reverse transcribe the mRNA using a *NotI*-d(T)₁₈ oligonucleotide which would be able to prime synthesis of the first strand cDNA provided that the PfTOP2 transcript(s) had been polyadenylated.

6.5. Reverse transcription of the PfTOP2 mRNA identifies sequence motifs that may cause premature transcriptional termination.

Total RNA derived from the induced PfTOP2 sample that had previously been used for northern blotting, was used for this experiment. SDS was removed from the samples by phenol/chloroform extraction and the RNA was recovered with ethanol precipitation. 10µg of RNA was treated with DNase I to remove any contaminating DNA from the sample. To ensure that the DNaseI was active, 0.8µg of plasmid DNA was treated and the reaction products monitored on an ethidium stained 1% agarose gel to confirm that the DNA had been completely degraded. DNase I was removed from the RNA samples by phenol/chloroform extraction, followed by ethanol precipitation of the RNA.

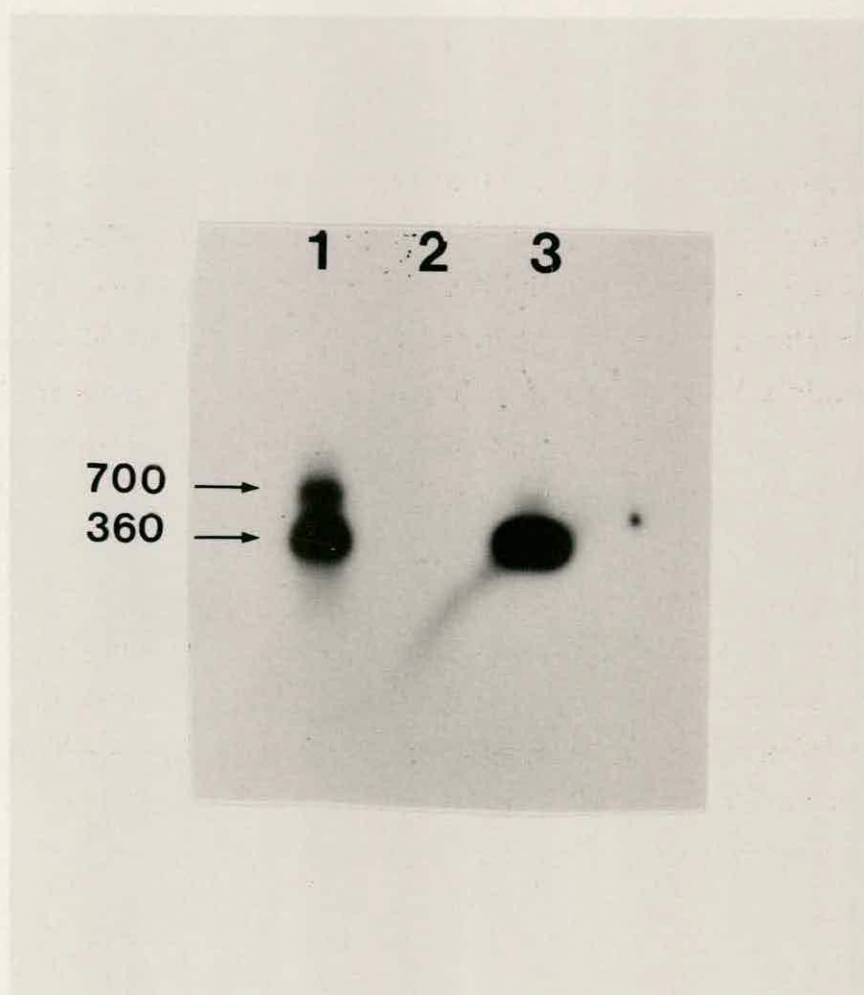
First-strand cDNA synthesis was achieved using the Pharmacia First-Strand cDNA synthesis Kit (described in section 2.4.4). This uses moloney murine leukemia virus (M-MuLV) reverse transcriptase to catalyse cDNA synthesis from a *NotI*-d(T)₁₈ primer, in the presence of dNTPs. Following synthesis of the cDNA, the resulting double-stranded DNA/RNA duplexes were amplified by PCR. This involved using the same *NotI*-d(T)₁₈ primer that was used to prime reverse transcription, and a PfTOP2-specific gene primer (719S). In a control reaction, the same primers were also used for PCR with a sample of the same RNA that had not been reverse transcribed. This was done to ensure that no DNA was present in the samples following DNase I treatment. A positive control was also used to ensure that the Taq polymerase and PCR parameters were functioning correctly. The template for this PCR was the PfTOP2 gene with PfTOP2 specific primers 719S and 288S which should give a PCR product of around 350 bp in size.

The products of the reverse transcriptase-PCR (RT-PCR) were Southern blotted, and a 5' [³²P] labelled PfTOP2 specific oligonucleotide (460S) which

Figure 6. (a)

Analysis of the products of reverse-transcribed RNA, (RT-PCR) using the PfTOP2 specific oligonucleotide 719(S) and the *NotI*-d(T)₁₈ primer that was originally used to prime synthesis of the cDNA. The PCR products were run out on a 1% agarose gel and stained with ethidium bromide. The gel was Southern blotted to Genescreen Plus membrane and probed with a 5' [³²P] labelled oligonucleotide 460(S) which spans nucleotides 319-340 of the PfTOP2 gene.

Lane 1 contains a PCR product derived from PCR amplification of the first strand cDNA. Lane 2 represents a PCR control, where total RNA isolated from galactose-induced cultures that was not reverse-transcribed was used as a template for a PCR. Lane 3 contains a 350 bp PCR product derived from amplification of the PfTOP2 gene.



spans nucleotides 319-340 of the gene was used to probe the blot. The results of this experiment are shown in figure 6. A major band of approximately 360 bp, along with a less abundant band of around 700 bp was detected by this probe; these were present only in the sample derived from the recombinant plasmid following galactose induction.

The PCR product was ligated into the TA pCRTTMII cloning vector (figure 7), transformed into INV α F' cells, and white colonies were picked and screened for the presence of recombinant plasmids. Although several attempts were made to isolate the 700 bp product, only the smaller, more abundant 360 bp product could be isolated from the transformation. Three clones from the transformation were analysed and their sequences are shown in figure 8. Polyadenylation sites were found to map to 370 (two clones, numbers 1 and 72) and 376 nt (1 clone, number 30) from the AUG. In addition, the poly (A) tails of the transcripts contained 15-18 adenine residues.

6.6. Replacement of putative transcription termination signals in the PfTOP2 gene.

An analysis of the region upstream of polyadenylation sites has revealed the presence of sequence motifs that have been demonstrated to act together to cause 3' end formation of yeast mRNA (Guo and Sherman, 1995). The 3' regions of seven genes in which major polyadenylation sites have been precisely mapped by sequencing are shown in Figure 9. Three interdependent, cis acting elements have been identified in *S. cerevisiae*; they are (i) the efficiency element, (ii) the positioning element and (iii) the actual poly (A) site. The efficiency element acts to enhance the function of the second element, the positioning element, sited downstream, which determines the actual site of polyadenylation. Polyadenylation often falls 16-27 nucleotides downstream of the positioning element and occurs preferentially after a pyrimidine base (figure 10).

An examination of the first 400 nt of the PfTOP2 gene reveals that several candidate efficiency and positioning elements occur within this region (shown in figure 8). In order to verify that these sequence motifs may be the cause of the premature transcription termination occurring at these

Map of the pCRTTMII TA Cloning vector.

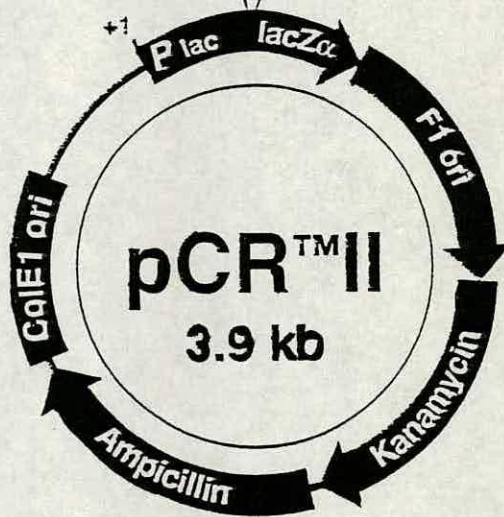
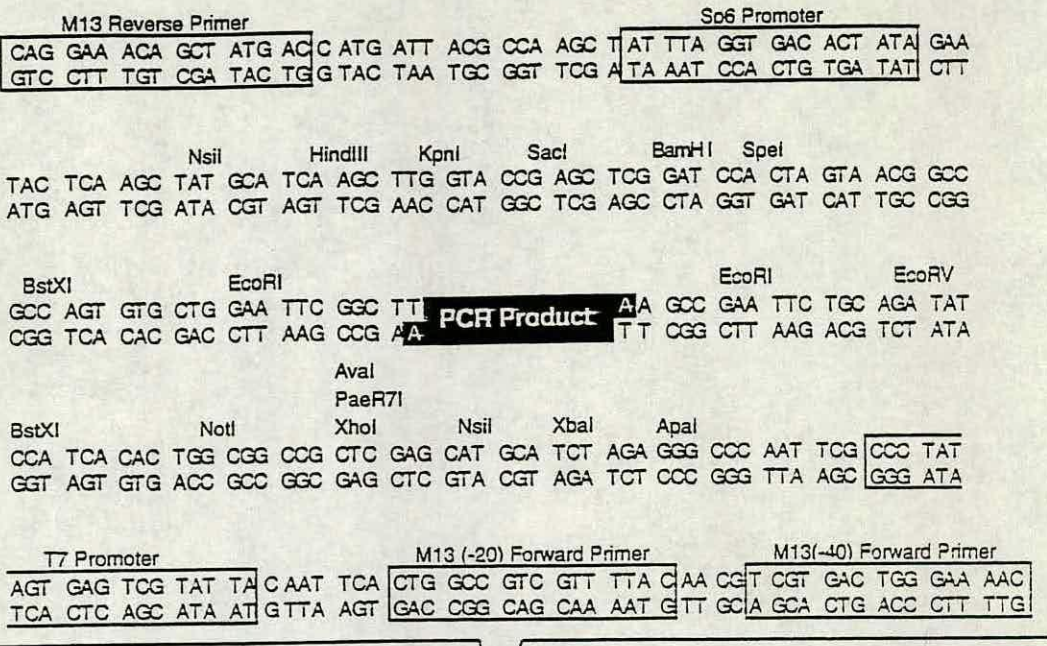


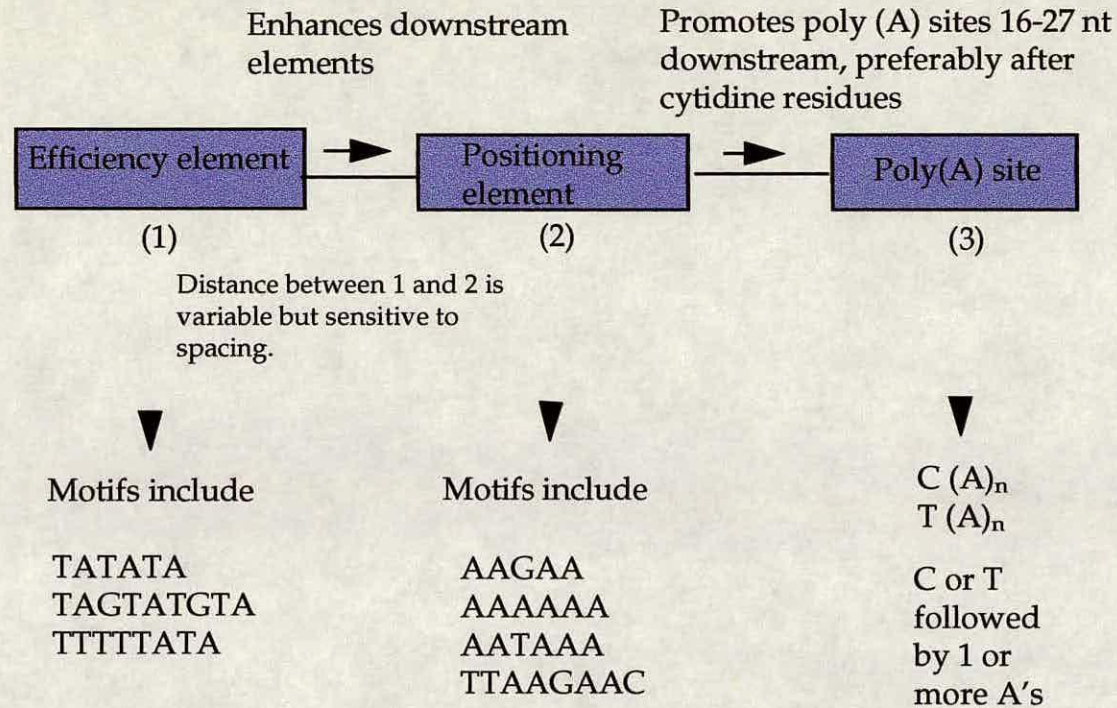
Figure 7. Map of the pCRTTMII TA Cloning vector.

	gtc	tac									gtc	gac	atc	cac						
agt	gta	tat	aat	gat	ggg	gaa	ggg	att	cct	gta	gat	gta	cat	aaa	gaa					
S	V	Y	N	D	G	E	G	I	P	V	D	I	H	K	E					
							▼			▼										
	aac	atc																		
atg	aat	ata	tat	gta	cca	cat	atg	att	ttt	gga	gaa	tta								
M	N	I	Y	V	P	H	M	I	F	G	E	L								

Figure 8.
Nucleotide sequence of the PFTOP2 gene showing the major polyadenylation sites that map at 370 and 376 nt from the AUG (shown as arrows). Also shown are the putative efficiency elements (boxed sequences) and the putative positioning element (italicised sequence). Sequences that were changed in an attempt to abolish premature transcription termination in the gene are shown above the boxed sequences.

Gene	Sequence
<i>ACT1</i>TAA....20...TGTT <u>TATGTATGTAC</u> CTCTCTCTCTATTTCTATTTTAAACCACCCTCTCAATAAAATAA ▼ AAATAATAAGATT'TTTAAGG	
<i>ADH1</i>TAA.....7...TCT <u>TATGATTTATGAT</u> TTTTATTATTAAATAAGTTATAAAAAAATAAGTGTATACAAATTT ▼ TAAAGTGACTCTTAGGTTTAA	
<i>CYC1</i>TAA...120...CCTATTTATTTTTT <u>TATAGTTATG</u> TTAGTATTAAGAACGTTATTTATTTCAAATT ▼	
<i>GAL7</i>TAA...100...AGTGACAATATATATATATATATATATATAATAATGACATCATTATCTGTAAAT ▼	
<i>GCN4</i>TGA....12...TTTATTTATATTTTTTATTTTCATTCTCGT <u>GTATAACGAAATAGAT</u> TACATTCAC ▼ TTAGATAAAGAAATTAATCTTTTTATGCCAATTT	
<i>MRP2</i>TAA.....14...AAAGTATATATATATATATAGGATACGCACTTGTAATAAATACATGATAATTTT ▼ TATTCTTCTTTAAT	
<i>RHO1</i>TAG...154...TCTTCTCTACATATAAGTGTATTGCTCAGTAAGTAATTATCATCTATTAAAG ▼	

Figure 9. Putative efficiency (-) and positioning (-) elements for 3' end formation of some yeast mRNAs. Arrows denote major poly (A) sites. Figure adapted from Guo and Sherman, 1995.



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Figure 10. Schematic representation of the elements involved in 3' end formation of yeast mRNA. (1) the efficiency element, (2) the positioning element and (3) the actual poly (A) site. Figure adapted from Sherman and Guo, 1995.

points in the PfTOP2 gene, we decided to change specific nucleotides within this sequence using PCR mutagenesis. The positions of the sites that were changed and their relationship with respect to the gene are shown in figure 11.

6.7. Northern analysis of the modified PfTOP2 sequence expressed from a GAL1 promoter in yeast.

Total RNA derived from induced cultures of the unmodified and modified PfTOP2 constructs were size fractionated and probed with the same PfTOP2 gene fragment (5' 1.8 kb, 1-1795 nt) that was used before to probe the original northern blot. Figure 12 shows the results of this experiment. Lane 1 contains RNA derived from the unmodified gene construct. A major transcript of around 360 nt can be seen; this transcript corresponds to the 370/376 nt transcripts identified by RT-PCR. Over-exposure of the membrane also reveals a minor transcript of around 650/700 nt in size. This signal could be interpreted to be derived from a transcript which may be the product of transcriptional read-through the major transcriptional block observed at 376 nt. In lane 2 (figure 12) a major transcript of around 600/700 nt can be seen, along with three very faint signals, the largest of which is about 2400 nt in length.

This result shows that the major transcriptional block in the unmodified PfTOP2 gene, was abolished by changing the putative efficiency elements as such: (i) tatata to tctaca, (ii) tagata to tcgaca, (iii) tacata to tccaca and (iv) tatata to catcta.

6.8 Identification of additional elements that may be responsible for premature transcriptional termination.

Total RNA from the same sample used for northern blotting (section 6.7) was used for this experiment after SDS from the storage buffer was removed by phenol/chloroform extraction, and ethanol precipitation. 10 μ g of RNA was treated with DNase I to remove any contaminating DNA from the sample. The activity of the enzyme was checked as described in section 6.5. of this chapter. DNase I was removed from the samples by phenol/chloroform extraction and ethanol precipitation.

Figure 11.

Replacement of putative transcription termination signals in the PfTOP2 gene.

In an attempt to abolish the premature transcription termination observed when the PfTOP2 gene is expressed from a GAL1 promoter in yeast, putative efficiency elements that occur within the gene sequence were mutationally replaced.

Figure 11 (a) shows the oligonucleotides (V7276 and V7277) that were used to introduce specified point mutations into the gene.

(b) shows the sequence of the PfTOP2 gene in the region where transcription termination has been demonstrated to occur, and the relationship of oligonucleotides V7276 and V7277 with respect to this sequence.

(c) shows a diagrammatic representation of the positions of all of the oligonucleotides that were used to amplify this region of the gene. As can be seen in Figure 1 (a), flanking oligonucleotides H4535 and G6165 made it possible to restrict the PCR product with *Kpn*I and *Pst*I so that the fragment could be cloned into YEplac181 in the steps described in Figure 1 (a) to (c).

Figure 11. Replacement of putative transcription termination signals in the PftOP2 gene.

(a) Sequence of the oligonucleotides that were used to modify the wild type PftOP2 sequence in an attempt to abolish premature transcription termination. Bold type denotes the base changes in the sequence.

5' CCTGT**CGACATCCACA**AGAAATGAAC**CATCT**ATGTAC 3'

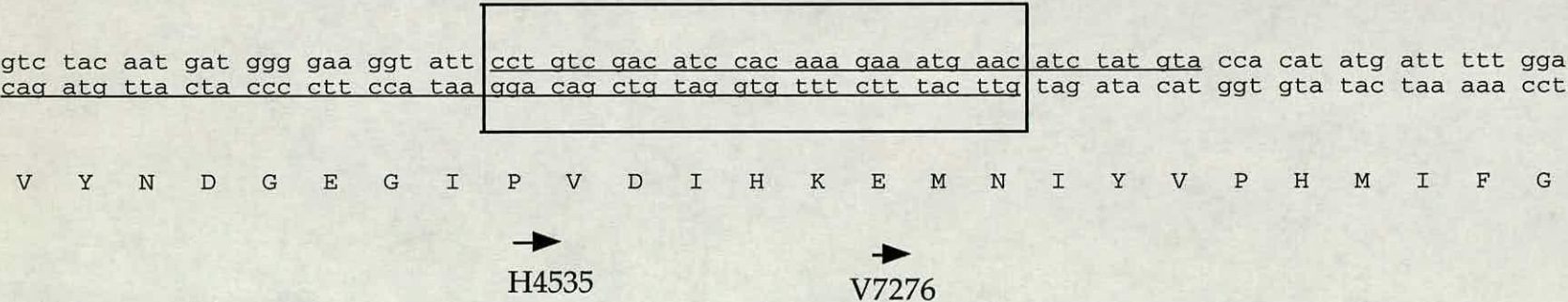
Sense strand sequence modifying oligonucleotide V7276.

3' CAGAT**G**TTACTACCCCTTCCATAAGGACAG**CTGTAGGTG**TTTCTTTACTTG 5'

Antisense strand sequence modifying oligonucleotide V7277.

(b) The sequence of the PftOP2 gene in the region where transcription has been demonstrated to terminate. Underlining denotes the sequence of the oligonucleotides that were used to incorporate silent point mutations that change the third base in a particular codon which is marked with a star. The boxed region denotes the region of homology between oligonucleotides V7276 and V7277.

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(c) Positions of amplifying oligonucleotides with respect to the template that was used for PCR

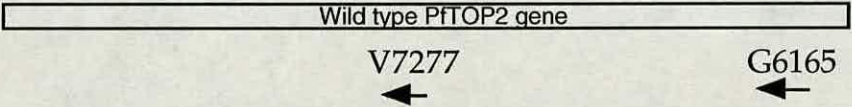
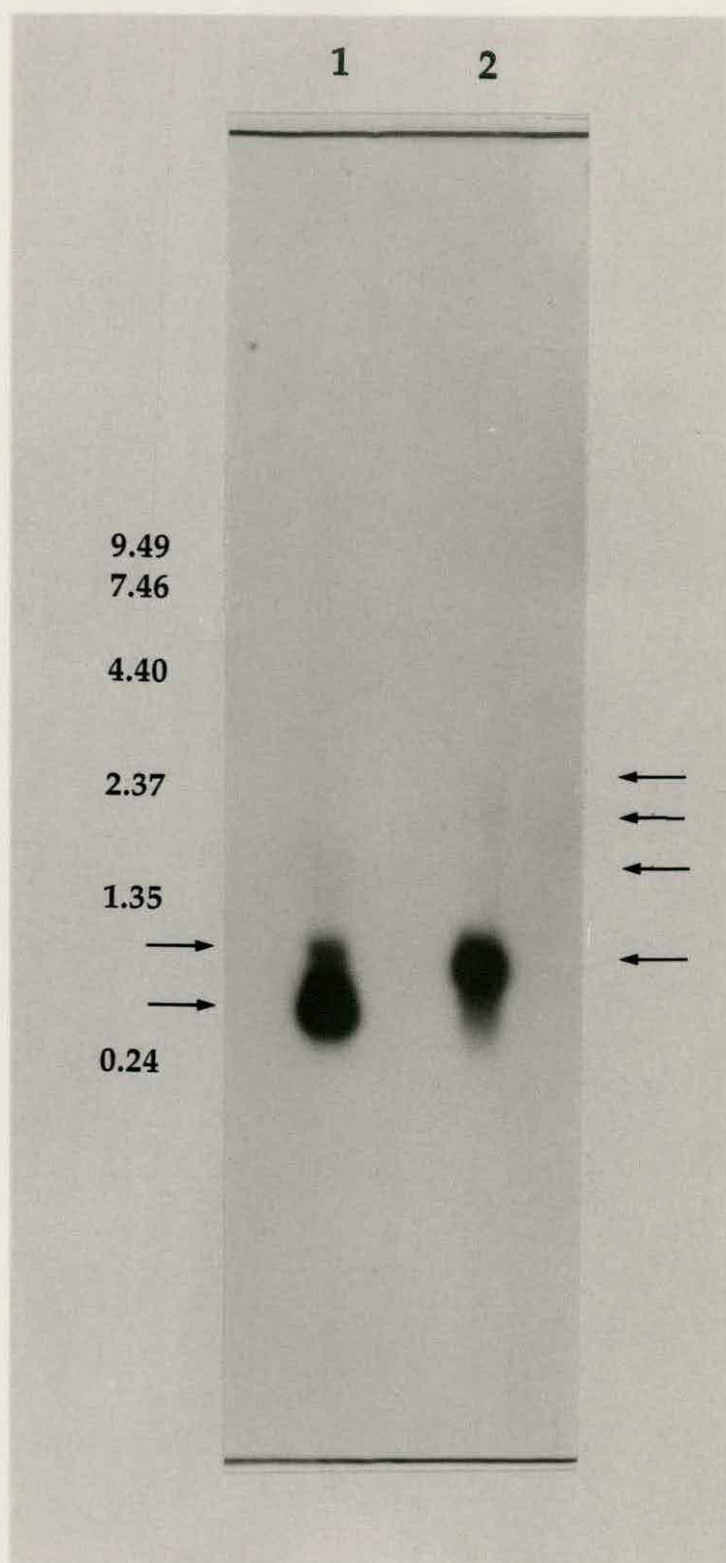


Figure 12.

Northern blot analysis of total RNA derived from yeast cultures containing the unmutated version of the PftOP2 gene, or a version of the gene where putative transcription termination sequences have been altered by PCR mutagenesis. The formaldehyde gel was blotted to Genescreen Plus membrane and probed with an 1.8 kb 5' fragment of the PftOP2 gene.

Lane 1 shows total RNA from a galactose induced culture containing the unmutated PftOP2 gene construct (YE*plac181*/PftOP2). Lane 2 shows total RNA from a galactose induced culture containing the mutated version of the GAL1/PftOP2 construct in YE*plac181*. The positions of the transcripts are arrowed, and the positions of the RNA size markers are shown on the left of the blot (sizes are in kilobases).



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First-strand cDNA sythesis was conducted using the same method as described in section 6.5 using the Pharmacia First-Strand cDNA synthesis Kit. The same *NotI*-d(T)₁₈ primer was used to prime synthesis of the first strand cDNA. RT-PCR was conducted as described previously (section 6.5) using the same *NotI*-d(T)₁₈ primer and sense PfTOP2 specific primer (719S). These primers were also used for RT-PCR using the same RNA which had not been reverse transcribed. This was done to ensure that no DNA was present in the samples following DNase I treatment. A positive control was also used as described in section 6.5.

The products of the reverse transcriptase-PCR (RT-PCR) were Southern blotted, and a 5' [³²P] labelled PfTOP2 specific oligonucleotide (460S) was used to probe the blot. This probe did not detect any of the bands that were present following agarose gel electrophoresis, which included a major band of approximately 700 bp, along with less abundant bands of around 500 and 300 bp (shown in figure 13) We cannot account for the failure of this probe to hybridise to any of the bands derived from the RT-PCR, but it is possible that the DNA did not transfer to the membrane efficiently. The PCR products were ligated into the TA pCRTTMII cloning vector (Invitrogen), transformed in INV α F' cells, and white colonies were picked and screened for the presence of recombinant plasmids. Several clones were obtained from the transformation but only two contained inserts. These were sequenced to map their 3' ends.

The nucleotide sequence of clone numbers 1 and 2 are shown in figure 14. Both clones terminate 617 bp from the ATG. An analysis of the sequence reveals several candidate efficiency and positioning elements.

Figure 13.

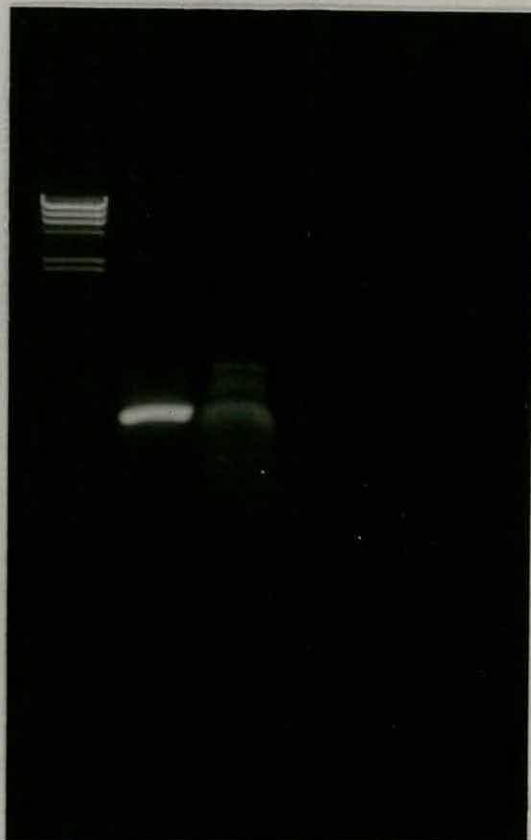
Agarose gel electrophoresis of PCR products. These were amplified from cDNA derived from reverse transcription of total RNA isolated from induced cultures containing the PfTOP2 gene in which the putative transcription termination sites had been altered.

Lane 1 shows the positions of the λ DNA cut with *Hind*III size standards (Gibco BRL) that were used. Their sizes are, from the top of the gel to the bottom; 23.15, 9.42, 6.56, 4.38, 2.32 2.02 and 0.56.

Lane 2 contains a 350 bp PCR product derived from amplification of the PfTOP2 gene. Lane 3 contains a PCR product derived from PCR amplification of the first strand cDNA. Lane 4 represents a PCR control, where total RNA isolated from galactose induced cultures that had not been reverse transcribed was used as a template for a PCR

The position of the 700 nt product is indicated.

1 2 3 4



6.9 Discussion.

Heterologous expression of PfTopoII with an N-terminal modification which replaced the first four *P. falciparum* codons with the first five yeast codons has been undertaken. This was done in the expectation that this modification would increase the likelihood of expression of the gene in yeast, in much the same way that a similar approach was successful for expression of human TopoII α (Wasserman *et al.*, 1993).

The N-terminal modified sequence was fused to a GAL1 promoter which could be regulated with galactose or glucose carbon sources. Originally, the intention was to subclone the GAL1/PfTOP2 cassette into the expression plasmid YEpWob6, but the construct was not maintained in any plasmid which contains a URA3 auxotrophic marker. This problem was circumvented by cloning the cassette into YEplac181 which contains a LEU2 auxotrophic marker instead. Initially, expression of the recombinant protein was monitored in two ways (i) induced cultures were lysed and size fractionated using SDS-PAGE. Polyacrylamide gels were stained with Coomassie blue and analysed for the presence of a novel band of around 160 kDa, the expected size of the recombinant protein. (ii) the same construct was transformed into t.s. yeast mutants lacking a functional TopoII, or TopoI and TopoII at restrictive temperatures, to determine whether expression of recombinant PfTopoII could rescue the defect(s).

Protein gels stained with Coomassie blue showed no evidence of expression of recombinant PfTopoII. However, induction of the human TopoII α positive control was observed, indicating that the conditions being used for expression were favourable. In addition to the absence of detectable protein, no evidence that expression of recombinant PfTopoII is able to rescue t.s. yeast strains that are defective in TopoII, or TopoI and TopoII function was observed; varying the amount of inducer used was also found to have no effect on cell growth at restrictive temperatures.

In order to investigate the lack of evidence of expression, we decided to look at steady state mRNA levels before and after induction of the protein. This would provide information on whether the GAL1 promoter was transcribing the gene. Northern analysis of total RNA showed that truncated PfTOP2 transcripts were produced. A major transcript of around

around 360 nt was observed. Prolonged exposure of the membrane also revealed a larger but less abundant transcript of approximately 700 nt in length. RT-PCR was used to map the 3' ends of these transcripts, but only the smaller cDNAs (370 bp and 376 bp) could be isolated.

The *P. falciparum* PfTOP2 gene, in line with many other genes of *P. falciparum* has an AT-content of over 74%, and although *S. cerevisiae* genes have, on average, 60% AT-content, we were concerned that this fundamental difference in gene structure could be detrimental to gene expression. Indeed, fortuitous transcription termination has been reported in other species with DNA of unusually high AT-content. For example, Romanos *et al.*, (1991) expressed the 1.5 kb Tetanus toxin fragment C (71%, AT-content) in *S. cerevisiae* and found that truncated transcripts were produced. Full length transcripts were only produced after resynthesising the entire gene to increase the G+C content from 29% to 47%. Romanos *et al.*, (1992) also report that the *P. falciparum* gene encoding p195 (0.88 kb) gives rise to multiple short mRNAs when expressed in *S. cerevisiae*.

Yeast mRNAs are processed at their 3' ends by endonucleolytic cleavage and polyadenylation to produce transcripts that end with a poly (a) tail of about 70 adenosine residues. Russo *et al.*, (1991) were the first to propose that specific cis acting motifs could be responsible for 3'-end formation of yeast mRNA. The efficiency element TATATA and related sequences, function to enhance the efficiency of a second element, the positioning element (AAGAA and related sequences) which in turn acts to position the actual site of polyadenylation. The fact that both of these elements are A+T rich increases the likelihood that these motifs may be present in the PfTOP2 gene, due to its high AT-content. The sequence of the PfTOP2 gene upstream of the site where premature transcription termination occurred was analysed to determine whether any candidate efficiency or positioning elements were present. Four candidate efficiency elements, and one positioning element were revealed in the sequence. The candidate positioning element falls within 16-27 nt of the 370 nt poly (A) site that is reported to be functionally relevant in the model proposed by Guo and Sherman (1995).

PCR mutagenesis of the putative efficiency elements within the PfTOP2 gene was used in an attempt to eliminate these elements and thus bypass the transcriptional block in the gene. Northern analysis of the mRNA produced from the modified gene revealed that an abundant longer transcript of approximately 600/700 nt was produced, along with other larger transcripts that could only be detected on overexposed blots. RT-PCR of this mRNA mapped the polyadenylation site to 617 nt from the AUG. Analysis of the sequence upstream of the Poly (A) site also reveals the presence of candidate transcription termination signals. These differ in sequence from the efficiency and positioning elements identified in the unmodified gene sequence, however, which illustrates the difficulties faced in removing such sequences from the coding sequence. It is clear that hitherto unidentified sequences are able to promote cleavage and polyadenylation. However, sorting them out, even by this method is likely to be time consuming and expensive.

It was mentioned in the introduction to this chapter that the DHFR and Pgh 1 proteins of *P. falciparum* have been used to complement t.s. mutations in the yeast DHFR gene and the *ste6* gene of *S. cerevisiae*. PfDHFR is a small gene (684 bp) encoding the 228 amino acid DHFR domain of the DHFR-TS enzyme. Analysis of the sequence reveals the presence of several candidate efficiency elements, such as one TATATA, and at least seven TATG motifs, in addition to at least 10 positioning elements (for example, a minimum of seven AAAAA and two AATAA motifs can be identified in the sequence). Recombinant PfDHFR has not been purified from yeast, and although expression levels have not been determined, it seems likely that the enzyme may not be expressed at high levels (Dr. Carol Sibley, *pers comm.*). The fact that transcript production has also not been measured, means that we do not know whether the presence of these sequences could act to reduce the overall level of protein production.

Ruetz *et al.*, (1996) have tried to purify recombinant PfPgh1 protein from *S.cerevisiae* t.s. mutants, but although they were able to demonstrate the presence of the protein by immunofluorescence staining of yeast transformants, they were unable to detect the protein on western blots.

Their interpretation of these findings are that the protein must have a very short half-life *in vivo*. PfPgh1 is a relatively large gene (4256 bp) which has an overall A+T content of 75% making it probable that candidate transcriptional termination sequences will be present. It would be interesting to know whether some degree of gratuitous transcription termination may be responsible for poor expression of PfPgh1, as is the case with PfTopoII.

Chapter 7

Chapter 7.

7.1 Discussion

The potential value of topoisomerase II as a target for antimalarial drugs, coupled with the difficulty of purifying the active protein from the parasite itself, made it desirable to isolate the coding sequence for the protein and attempt to express it in a more amenable cell system. We chose to use expression systems that are routinely employed for expression of foreign proteins, such as *Escherichia coli*, the baculovirus/insect cell system and *Saccharomyces cerevisiae*, in the hope that with one or more of them a sufficiently high level of active protein might be produced to permit biochemical studies and drug tests to be performed.

7.2 Heterologous expression in *E. coli*.

Extensive knowledge of the genetics of *E. coli* have made it a valuable tool for the expression of foreign DNA sequences. However, not every sequence can be expressed in this system. Numerous factors influence whether a foreign DNA sequence will be expressed. Some of the reasons why a particular recombinant protein is not produced in high yield are, for example, (a) subtle differences between the structural features of the foreign gene and the genes of the bacterium, (b) the mRNA may be unstable, (c) there may be differences in codon usage between *E. coli* and the organism from which the foreign DNA is derived, (d) the recombinant protein may be expressed, but may not fold correctly resulting in proteolysis, and (e) the recombinant protein may be toxic to the cell. One of the major obstacles to functional heterologous expression in *E. coli* relates to the inability of the system to perform the post-translational modifications characteristic of many eukaryotic proteins.

Although it did not prove possible to purify full length recombinant PfTopoII from *E. coli*, there is evidence to suggest that the protein, or part of it, may have been expressed. Western blots derived from induced cultures containing the PfTOP2 ORF as a fusion to GST, displayed

numerous GST-cross reactive proteins that were less than 100 kDa in size. Our failure to detect full length recombinant protein could indicate that the protein is toxic to the cells, however, if that is the case, then it is clear that toxicity is not dependent on the functionality of the protein.

Reasonable levels of expression of the N-terminal (599 amino acid) and C-terminal (404 amino acid) regions of PfTopoII were observed, suggesting that the failure of expression of full length PfTopoII was not caused by the structural peculiarities of the gene, or differences in codon usage at least.

7.3 Heterologous expression using baculovirus.

As an expression system, baculovirus has gained considerable recognition during the 1990s. This is particularly so for the expression of eukaryotic proteins where biological activity is the primary consideration. Insect cells are believed to execute many of the post-translational modifications that are believed to be important for full biological activity, such as phosphorylation, glycosylation, and signal peptide cleavage. Furthermore, disulphide bond formation and protein folding are also reported to be performed more readily in this system.

There are several factors that may adversely affect heterologous expression of a foreign gene in baculovirus, including, (i) the presence of introns, (ii) the context of the AUG, (iii) the presence of 5' and 3' untranslated regions in the gene, and (iv) the presence of TAAG and CTTA sequence motifs in the foreign gene.

Immunologically detectable quantities of recombinant PfTopoII were produced in baculovirus, but the yields were poor. The reasons for this were not immediately obvious. There are no introns, or 5' and 3' untranslated regions present in the PfTopoII construct. Moreover, the context of the AUG in the wild type PfTOP2 gene was modified to conform to a consensus sequence that appears to be preferred for expression by those baculovirus genes for which information is available. However, analysis of the PfTOP2 mRNA transcribed by baculovirus revealed numerous truncated transcripts which suggests that TAAG and CTTA signals may be influencing the level of full length transcription. Inspection of the PfTOP2 gene sequence revealed at least 19 TAAG

sequence motifs on the sense strand. TAAG and CTTA sequence motifs are known to specify late and very late transcript initiation sites in baculoviral genes, in the sense and antisense directions, respectively. The PfTOP2 gene was expressed from the late polh promoter, and this may have increased the possibility that the TAAG/CTTA motifs within the gene were "seen" more readily as transcription initiation sites. This unfortunate but interesting possibility has been remarked on by Dr. R.D. Possee (Institute of Virology and Environmental Microbiology - Oxford) as being the most likely cause of low level expression of the PfTOP2 gene (*pers comm.*). It would certainly be interesting to see whether the removal of these sequence motifs improves the expression levels of the gene.

An additional problem encountered with the system was revealed when attempts to purify the recombinant protein were made. Although immunologically detectable amounts of the full length protein were purified using a nickel affinity column, a co-purifying host cell TopoII activity was present. It may have been possible to remove this contaminating activity if larger quantities of PfTopoII has been made. Rossi *et al.*, (1996) have purified recombinant human TopoI from baculovirus using a similar approach. Although the recombinant enzyme was expressed at high levels (4 mg from 10^9 infected cells) and purified by nickel column chromatography, Rossi *et al.*, report that trace amounts of a co-purifying insect cell TopoI are likely to be present.

7.4 Heterologous expression in *S. cerevisiae*.

Yeast is an attractive system for expression of foreign genes. It combines well defined genetics, ease of handling and availability of temperature sensitive mutants, with inexpensive conditions for growth. But not all foreign sequences express well in yeast. Many of the factors that influence the level of expression of a foreign gene in *E. coli* are also common to yeast (discussed above in a to e). Differences in codon usage, for example, may affect expression of foreign proteins, but other *P. falciparum* genes, such as DHFR have been successfully expressed in *S. cerevisiae* indicating that this may not be a problem.

Although human TopoII α and β have been expressed in yeast, expression of recombinant PfTopoII was not observed. However, we have been able to determine at least one reason, possibly the main one, why this is the case. Because of the relatively high (74%) A+T content of the PfTOP2 sequence, it contains within it gratuitous AT-rich elements that are known to direct 3' end formation of yeast mRNA. These elements, which are known as the efficiency and positioning elements were suspected of causing premature transcription termination in the PfTOP2 gene. The fact that the transcriptional blocks could be abolished by mutagenesis directed at specific sites within the sequence confirms this. Other potential transcription termination signals are clearly present downstream of the second transcriptional block in the PfTOP2 sequence, and although only some of these may be present in the right context to cause transcription termination, their frequent occurrence, and the fact that we may not be able to identify all of them, precludes the possibility of changing all of the sequences involved individually by site directed mutagenesis.

An alternative approach, chemical synthesis of the entire gene, to remove all candidate transcription termination elements and to increase the G+C content, may be the way forward to achieving full length expression of the PfTOP2 gene in yeast.

7.5 Expression of PfTopoII throughout the blood stages of parasite growth.

An analysis of the stage-specific expression of PfTopoII was also conducted using parasites that had been synchronised in ring, trophozoite or schizont stages. PfTOP2 transcripts were found to accumulate principally in the trophozoite stage parasite, although nuclear run-on analysis (conducted by Paul Horrocks) revealed that the promoter was active at all developmental stages.

TopoII protein accumulated predominately in trophozoites and schizonts, and at similar levels, whilst enzyme activity, as measured by decatenation of KDNA, reached a peak at schizogony.

These findings provide the basis for future work to examine in greater detail how expression of PfTopoII is regulated, and the ability of TopoII poisons to act selectively against the parasite enzyme, thus providing new

7.6 Future work.

Heterologous expression in *S. cerevisiae*.

(i) Reconstruction of the gene for PfTopoII would involve designing 100-mer oligonucleotides, which, whilst preserving the unique amino acid sequence of the wild type gene, will introduce specific changes into the DNA sequence to increase the G+C content and eliminate the mRNA cleavage and polyadenylation sites. Alignment of the wild type PfTOP2 DNA sequence with the proposed synthetic sequence shows that considerable stretches of identity remain. This makes it possible to use the antisense strand of the wild type gene (isolated from M13) as a scaffold with which to align each 100-mer oligonucleotide by hybridisation. This process could be conducted using five 100-mer oligonucleotides which can be ligated together to make a 500 nt segment of gene which can be amplified by PCR. Eight segments, each of around 500 nt, will be PCR amplified in this way to make up the entire synthetic gene. Each segment will contain unique restriction sites to facilitate cloning, and, after sequencing to ensure that the synthetic sequence is correct, the final product can be expressed in yeast.

Unfortunately, shortage of time precluded this plan from being fully implemented, but it will be pursued in future in the hope that expression and complementation of the *top2* defect in yeast will be achieved.

(ii) Use of alternative expression systems, such as the slime mold *Dictyostelium discoideum* may also be worth consideration in the future. The DNA of this organism is highly A+T biased and as such may be useful for expression of the A+T rich PfTOP2 gene. Fasel *et al.*, (1992) have expressed the circumsporozoite protein of *P. falciparum* in *D. discoideum* and found that the natural characteristics of the protein (as judged by size and epitope recognition) were retained.

(iii) Another approach would involve the use of established methods which are able to detect cleavable complex formation *in vivo* following treatment of the parasite with topoisomerase II poisons which are known to act via this mechanism. This approach would bypass purification of the PfTopoII enzyme and the difficulties associated with it.

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